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ORIGINAL SUBMISSION

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Lonza

SENT *VIA* FEDEX

January 6, 2010

Robert L. Martin, Ph.D.
Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition
Food And Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740-3835

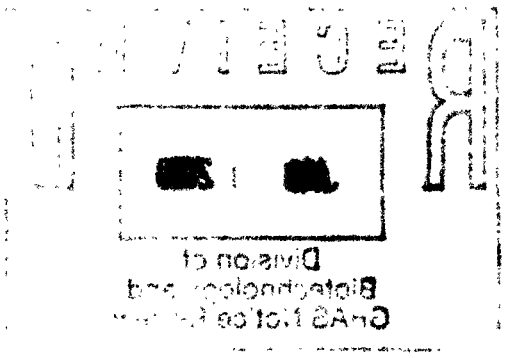
Re: GRAS Notice for Ulkenia DHA oil derived from *Ulkenia* sp. microalga

Dear Dr. Martin:

In accordance with proposed 21 CFR §170.36 [Notice of a claim for exemption based on a Generally Recognized As Safe (GRAS) determination] published in the *Federal Register* [62 FR 18938 (17 April 1997)], I am submitting in triplicate, as the notifier [Lonza Ltd. Muenchensteinerstrasse 38 CH-4002 Basel, CH-4002 Switzerland], a Notice of the determination, on the basis of scientific procedures, that Ulkenia DHA oil derived from *Ulkenia* sp. microalga has been determined by Lonza Ltd. to be Generally Recognized as Safe (GRAS), consistent with Section 201(s) of the Federal Food, Drug, and Cosmetic Act and therefore, is exempt from the premarket approval requirements of the *Federal, Food, Drug and Cosmetic Act.*

Information setting forth the basis for the GRAS determination, which includes a comprehensive summary of the available data, has been reviewed by an independent panel of experts (the Expert Panel), qualified by scientific training and experience to evaluate the safety of Ulkenia DHA oil in traditional foods. The current GRAS notice is in follow-up to GRAS notice 000160 which was withdrawn upon the request of Nutrinova Nutrition Specialities & Food Ingredients GmbH (Nutrinova) in a letter sent to the Agency in March 2005. Since that time a number of significant events have occurred which has impacted upon the notification that is now being submitted. The first of these changes relates to the purchase of the DHA product from Nutrinova by Lonza Ltd, hence the change in the address and contact information within the current notification. As a consequence Lonza has subsequently amended the name of the ingredient within the notification from docosahexaenoic acid (DHA)-rich triacylglycerol oil derived from *Ulkenia* sp. microalga to Ulkenia DHA oil derived from *Ulkenia* sp. microalga. A further significant event during the intervening years has been the untimely death of one of the Expert Panel members, Professor Kroes, who was involved with the GRAS determination.

The Expert Opinion statement attached to the notification has as a result been updated to include the changes referred to above, as well as indicating that no information has come to light through November 2009 which would alter the opinion of the remaining Panel members regarding the original safety assessment of Ulkenia DHA oil under the proposed conditions of intended use. The two surviving Expert Panel members have resigned the Expert Opinion statement further endorsing the safety of Ulkenia DHA oil under the proposed conditions of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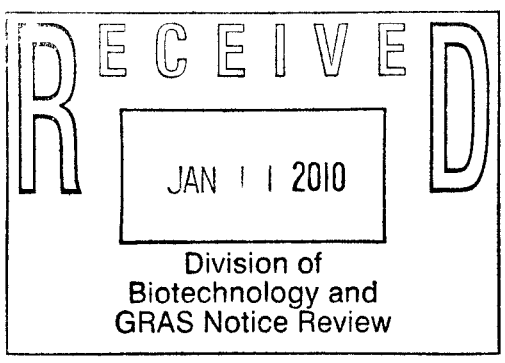
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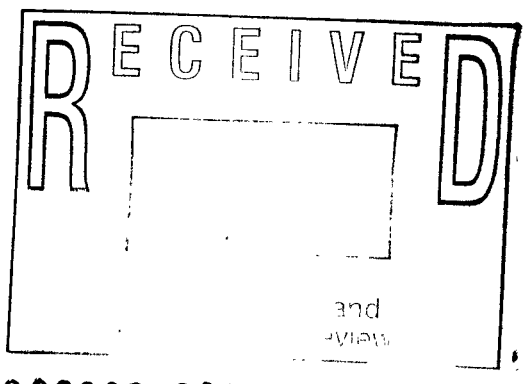
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intended use. I have also included the original signature page containing all three Expert panel members. I trust that the enclosed Notice and the changes made to the Expert Opinion statement meets with the Agency's approval. Should you have any questions or concerns regarding this amended GRAS Notice (replacing GRN 000160), please do not hesitate to contact me at any point during the review process so that we may provide a response in a timely manner.

Sincerely,

Rene Blum Ph.D.
Sr. Manager Scientific and Government Affairs

GRAS NOTICE FOR ULKENIA DHA OIL DERIVED FROM *ULKENIA* SP. MICROALGA

Prepared for:

Robert L. Martin, Ph.D.
Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition
Food And Drug Administration
5100 Paint Branch Parkway
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Prepared by:

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January 6, 2010

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GRAS NOTICE FOR ULKENIA DHA OIL DERIVED FROM *ULKENIA* SP. MICROALGA

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I. GRAS EXEMPTION CLAIM

A. *Claim of Exemption From the Requirement for Premarket Approval Pursuant to Proposed 21 CFR §170.36(c)(1) [62 FR 18938 (17 April 1997)]*

As defined herein, docosahexaenoic acid (DHA)-rich triacylglycerol oil derived from *Ulkenia* sp. SAM2179, a thraustochytrid microalgae (Ulkenia DHA oil) has been determined by Lonza Ltd. (Lonza), to be Generally Recognized as Safe (GRAS), consistent with Section 201(s) of the Federal Food, Drug, and Cosmetic Act. This determination is based on scientific procedures as described in the following sections, under the conditions of its intended use in food. Therefore, the use of Ulkenia DHA oil in food as described below is exempt from the requirement of premarket approval.

Signed,

Dr Rene Blum
Lonza Ltd.
Muenchensteinerstrasse 38
CH-4002
Basel, CH-4002
Switzerland

Date January 6, 2010

B. Name and Address of Notifier

Dr Rene Blum
Lonza Ltd.
Muenchensteinerstrasse 38
CH-4002
Basel, CH-4002
Switzerland

C. Common Name of the Notified Substance

Ulkenia DHA oil

D. Conditions of Intended Use in Food

Ulkenia DHA oil is intended for use as a food ingredient to increase the intake of dietary ω-3 polyunsaturated fatty acids (PUFAs), particularly DHA, a critical component of most cell membranes and tissues. The individual intended food use levels of Ulkenia DHA oil in food,

discussed in greater detail in Section IV.B herein, are consistent with the current GRAS use of menhaden oil specified in 21 CFR §184.1472 and the revised uses outlined in the U.S. Food and Drug Administration (FDA)'s final rule regarding GRAS-affirmed food uses for menhaden oil (U.S. FDA, 2005), respectively.

E. Basis for the GRAS Determination

Pursuant to 21 CFR § 170.30, the composition of Ulkenia DHA oil intended for use in foods by Lonza, as defined in Table 4, has been determined to be GRAS based on scientific procedures (U.S. FDA, 2008). This determination is based on data generally available in the public domain pertaining to the safety of DHA and supported by the historical consumption of DHA from fatty fish (*e.g.*, haddock, tuna, salmon, mackerel) and other food sources as discussed herein and in the accompanying documents, and is supported by a consensus among a panel of experts who are qualified by scientific training and experience to evaluate the safety of Ulkenia DHA oil as a component of food[1] [see Appendix, Tab 1, entitled, "**EXPERT PANEL CONSENSUS STATEMENT: THE GENERALLY RECOGNIZED AS SAFE (GRAS) STATUS OF ULKENIA DHA OIL UNDER THE CONDITIONS OF INTENDED USE IN TRADITIONAL FOODS**"].

F. Availability of Information

The data and information that serve as the basis for this GRAS Notification will be sent to the FDA upon request, or will be available for review and copying at reasonable times at the offices of:

Lonza Inc.
17-17 Route 208
Fair Lawn, NJ 07410
USA

Should the FDA have any questions or additional information requests regarding this notification, Lonza will supply these data and information.

II. DETAILED INFORMATION ABOUT THE IDENTITY OF THE SUBSTANCE

A. Identity

Ulkenia DHA oil is a refined triacylglycerol oil (>95% triacylglycerols), derived from Ulkenia sp., a marine protist, with a total fatty acid composition of 38 to 50% DHA. The remaining fatty acids of Ulkenia DHA oil are comprised mainly of saturated palmitic acid (16:0) (28 to 37%) and a lesser amount (8 to 14%) of the ω-6 fatty acid, docosapentaenoic acid (DPA) (22:5). In

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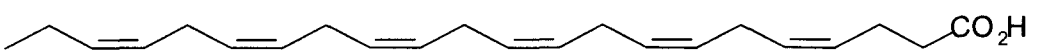
[1] The Panel consisted of Robert Kroes, D.V.M., Ph.D. (Utrecht University), Ernst Schaefer, M.D. (Tufts University), and Gary Williams, M.D. (New York Medical College).

addition, Ulkenia DHA oil contains safe and suitable antioxidants as permitted by the FDA for use in edible oils to ensure stability.

Common or Usual Name:	Ulkenia DHA oil, containing 38 to 50% DHA; DHA oil 45; DHA45-TG, DHAid, Lonza DHA, DHAid™
Chemical Name:	All-cis-4,7,10,13,16,19-docosahexaenoic acid (22:6) esterified to glycerol
Chemical Abstracts Service	None assigned for DHA-rich oil; however, DHA assigned CAS No. 6217-54-5

The structural formula of DHA is provided below in Figure 1.

Figure 1 All-cis-4,7,10,13,16,19-docosahexaenoic acid (22:6)



B. Method of Manufacture

Ulkenia DHA oil is manufactured in accordance with current good manufacturing practices through a multi-step fermentation and refining process using a genetically stable and pure culture of the marine protist, *Ulkenia* sp. Ulkenia DHA oil is extracted from the dried biomass *via* a pressing step or directly from wet biomass by cell rupture and oil separation. Optional, a solvent-based extraction process using food grade solvents and processing aids can be used. The extracted crude oil is further refined by processes standard to the edible oil industry, including degumming, deacidification, bleaching and deodorization, using materials appropriate for food processing. Ulkenia DHA oil can be mixed with other edible oils like sunflower oil *etc.*, to adjust the DHA concentration to a specified content to allow convenient use. By using common oil processing technology like winterization, the DHA content can be further increased and the content of saturated fatty acids can be decreased. Additional information is provided below.

C. Raw Material Specifications

a *Ulkenia* sp.

Ulkenia DHA oil is produced through a multi-step fermentation and refining process using a non-modified, wild type marine microalgae, *Ulkenia* sp. The production organism is deposited as SAM2179 at the Agency of Industrial Science and Technology, Japan. It is also described in

patent WO 98/03671. Additional information related to the source organism is provided in Appendix (Tab 2).

b Culture Medium

The production medium used in the manufacturing process for Ulkenia DHA oil is based on glucose and corn steep (GC). It contains the following ingredients: dextrose monohydrate or glucose syrup, potassium phosphate monobasic (KH_2PO_4), sodium chloride (NaCl), magnesium sulfate ($MgSO_4$. $7H_2O$), calcium chloride ($CaCl_2$ $2H_2O$), corn steep liquor, ammonium sulfate $(NH_4)_2SO_4$, and optional an antifoaming agent. In addition, phosphoric acid (H_3PO_4) and potassium hydroxide (KOH) or sodium hydroxide (NaOH) are used as pH adjusting agents prior to sterilization and during fermentation. All components of the culture medium meet food grade specifications or are of adequate purity for food fermentation processes. Safe and suitable antifoaming agents, meeting appropriate specifications, are used during fermentation steps in the production of Ulkenia DHA oil.

One such antifoaming agent is a copolymer of ethylene oxide (EO) and propylene oxide (PO). This material is approved as a direct food additive, defoaming agent up to 0.05% by weight in 21 CFR Sec. 172.808 (U.S. FDA, 2008). While, the list of approved uses included in this section does not include the manufacture of edible oils, the fact that this product is an approved defoaming agent supports its use in the production of Ulkenia DHA oil for use as a food ingredient. Furthermore, Lonza indicates that the typical level of use of this copolymer antifoam is 15 ppm in the fermentation broth, up to a maximum of 40 ppm. The use of this antifoam agent is many processing steps away (or distant) from the final product of refined Ulkenia DHA oil and residues of the antifoam agent would not be expected to remain in the final product. The water-soluble copolymer condensate of ethylene oxide and propylene oxide would not be expected to be retained in the oil following solvent extraction with hexane and any traces would be removed during subsequent bleaching steps. According to the manufacturer's specifications, the maximum concentrations of EO and PO are 0.5 and 1 ppm, respectively, as residual monomers in the copolymer condensate antifoam. Thus, under the intended conditions of use as an antifoam agent, worst-case maximum exposure to these monomers would be *de minimis* and of no toxicological concern.

Another such antifoaming agent is linear dimethylpolysiloxane solution containing small levels of surfactants including sorbitan esters of fatty acids, glycerin esters of fatty acids and sucrose esters of fatty acids. Dimethylpolysiolxane is considered to be GRAS for use as a direct food additive at levels of up to 10 ppm in food in accordance with 21 CFR 173.340 (U.S. FDA, 2008). The surfactants are either GRAS or approved as additives or defoaming agents under 21 CFR 172.842, 184.1323, or 172.859 and 21 CFR 173.340 (U.S. FDA, 2008).

c Flocculating Agents

Chitosan is considered a safe and suitable flocculating agent that may be used during the extraction phase of the manufacturing of Ulkenia DHA oil. The acceptability of its use within the GRAS determination for Ulkenia DHA oil for use as a food ingredient is further discussed herein.

Chitosan is a modified natural carbohydrate polymer derived from chitin, which occurs principally in animals of the phylum Arthropoda. The primary unit in the chitin polymer is 2-deoxy-2-(acetylamino)glucose. These units are combined by 1-4 glycosidic linkages, forming a long chain linear polymer. Removal of most of the acetyl groups of chitin by treatment with strong alkalis yields chitosan (Peniston and Johnson, 1980).

The chitosan intended for use as a processing aid in the production of Ulkenia DHA oil would be used as a flocculating agent used during the extraction phase. Subsequent refinement processes to the final product of Ulkenia DHA oil would remove all traces of chitosan. Consumers are already exposed to this material through its use in other applications. Chitosan is used as a dietary supplement, as an excipient for oral drug formulations, wound dressings, and cosmetics. It is also used as a preservative coating and biofungicide when sprayed on fresh fruits and vegetables and fertilizers. A dieter intentionally ingesting chitosan capsules and unintentionally ingesting chitosan as an excipient in a medication, as an ingredient in low-fat dairy products, and as a preservative sprayed on fruits can easily consume a daily dose of 10 to 20 grams (NTP, 2004).

Relevant studies examining the safety of chitin and chitosan were identified in the published literature. Kim *et al.* (2001) administered Sprague-Dawley rats doses of 500, 1,000, and 2,000 mg/kg/day chitosan oligosaccharide by gavage daily for 4 weeks. No significant differences in behavior or external appearance, body weight and food consumption between control and treated rats were observed. In addition, no significant differences in urinalysis, hematology, blood biochemistry, relative organ weights, and histopathological findings were found in both control and treated rats. Thus, the no-observed-adverse-effect level (NOAEL) of chitosan oligosaccharide was considered to be over 2,000 mg/kg in rats. Similarly, Shigamatsu *et al.* (2001) reported no effects on hematological or blood chemistry parameters, organ weights (heart, liver, and kidney) or histopathological changes in the liver and kidneys of male Wistar rats administered 8 mg/kg/day chitosan for 10 weeks. Niho *et al.* (1999) reported no obvious signs of toxicity when chitin was administered to F344 rats at concentrations up to 5% in the diet for 13 weeks.

In comparison, the use of chitosan as a flocculating agent during the extraction of oil and its subsequent removal from the finished product would result in *de minimis* worst-case maximum exposure and such exposure would be of no toxicological concern

d Hexane

Hexane as an option may be used as a solvent for crude oil extraction from *Ulkenia* sp. biomass and as a processing aid during refining of the oil. Hexane, meeting appropriate food grade specifications, is approved for food additive use in the production of spice oleoresins and hops extract in accordance with 21 CFR §173.270 (U.S. FDA, 2008). It is also listed as a permissible residue in various food ingredients under certain conditions (*e.g.*, fish protein isolate, cocoa butter substitute) (21 CFR §172.340, 184.1259) (U.S. FDA, 2008).

As outlined in the manufacturing process, a portion of crude Ulkenia DHA oil could be extracted from the fermentation biomass using hexane, which is subsequently removed by vacuum distillation. Hexane could further be used in the refining process, prior to deodorization of Ulkenia DHA oil final product, and removed by evaporation. No traces of hexane (<1 ppm) were detected in 3 non-consecutive lots of Ulkenia DHA oil using GC headspace analysis. The results for hexane analyses of 3 non-consecutive hexane extracted representative lots of Ulkenia DHA oil are presented in Table 1.

Table 1 Hexane Residue in Ulkenia DHA Oil

Residue	Method	Lot 990222	Lot 110708	Lot 120306
Hexane (ppm)	DIN EN ISO 9832	<1	<1	<1

e Degumming Acids

Aqueous solutions of food acids, meeting appropriate food grade specifications, especially acetic, phosphoric or citric acid, are used as degumming agents in the manufacturing process of Ulkenia DHA oil (21 CFR §184.1005, 21 CFR §182.1073, and 21 CFR §184.1033, respectively) (U.S. FDA, 2008).

f Neutralizing Agent

Dilute aqueous solutions of sodium or potassium hydroxide, meeting appropriate food grade specifications, are used to remove any free fatty acids in the manufacturing process of Ulkenia DHA oil (21 CFR §184.1631, 21 CFR §184.1763) (U.S. FDA, 2008).

g Bleaching Agents

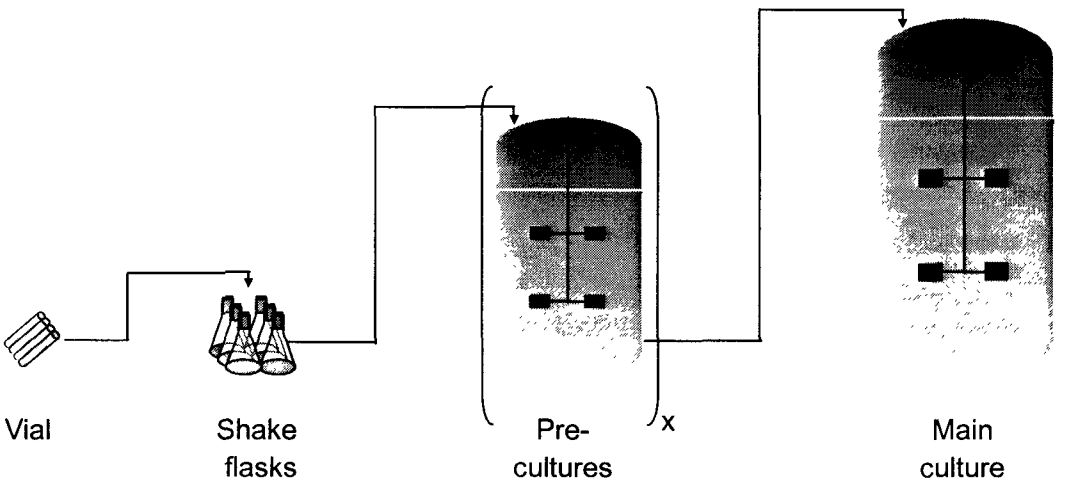
Bleaching clay and activated carbon, of appropriate food-grade specification, are used as the bleaching agents during the refining of crude Ulkenia DHA oil.

D. Manufacturing Process for Ulkenia DHA Oil

a Fermentation

The production of Ulkenia DHA oil commences with large-scale fermentation of *Ulkenia* sp., as presented in Figure 2. Pre-culture flasks are shaken and inoculated with one vial of *Ulkenia* sp. Lonza maintains the genetic stability, cultural purity, sterility, and integrity of the microalgae, *Ulkenia* sp. Before and after each fermentation step, samples of the pure strain, and samples of shake flask culture, pre-culture, and main culture, are checked on Petri dishes with nutrient agar for the detection of microbial contamination. The culture is transferred to the first seed fermentor (pre-cultures). This culture is subsequently transferred to additional seed fermentors until the volume is sufficient for inoculation of the main fermentor (main culture).

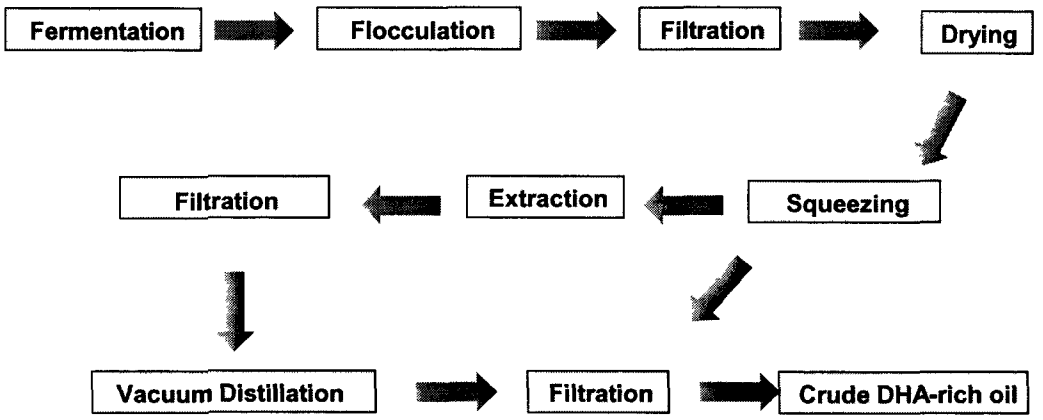
Figure 2 Schematic Overview of the Fermentation Process for Ulkenia DHA Oil



b Extraction and Refining

Following fermentation, Ulkenia DHA oil is extracted from the biomass as follows in Figure 3. The fermentation broth is cooled and flocculated by adding suitable flocculating agents. The flocculated broth is then filtered and the filter cake is dried. The dry biomass can be squeezed (pressed) to obtain crude oil directly (press oil). The squeezed biomass may additionally be extracted using hexane to obtain the residual oil (sep oil). Both fractions could be mixed or held separate. After filtration any solvents used are removed from the miscella using vacuum distillation. Alternatively, Ulkenia DHA crude oil can be directly obtained from wet biomass by cell rupture and oil separation, eliminating drying and any solvent extraction steps. The retained oil is then filtered to obtain crude Ulkenia DHA oil.

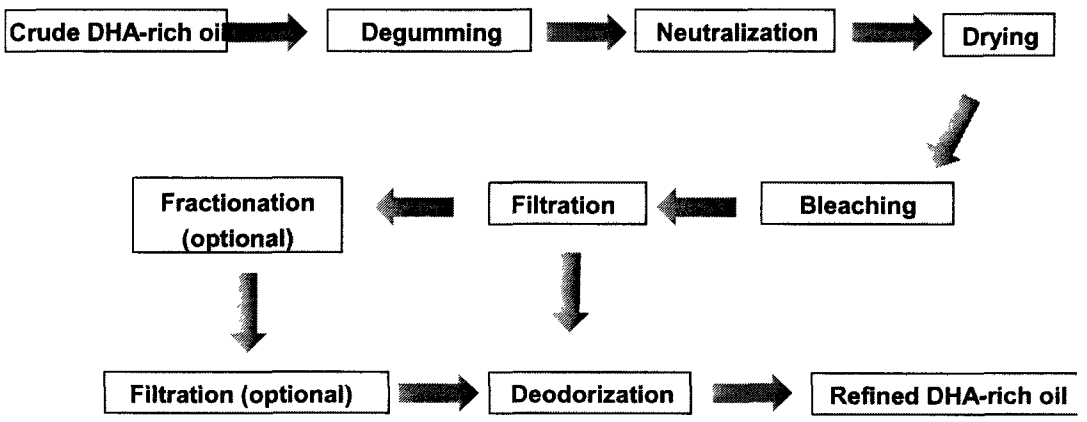
Figure 3 Production of Ulkenia DHA Oil from Fermentation Biomass



The crude Ulkenia DHA oil is refined *via* a standardized food-oil refining process to a final food-grade product, as shown in the following Figure 4. The crude Ulkenia DHA oil is degummed by adding aqueous food acid to remove traces of phospholipids. The organic and inorganic phases are separated by centrifugation and the organic phase is neutralized. The degummed neutralized oil is bleached using clay. The suspension is filtered to remove residual clay. The oil might be fractionated (*e.g.* winterized) to remove higher melting fats by filtration. The bleached oil is deodorized by hot water steam that is driven through the oil. The final product is refined Ulkenia DHA oil, which is stabilized by addition of safe and suitable antioxidants.

Refined Ulkenia DHA oil is stored and transported in vessels suitable for food storage under nitrogen at ≤5°C.

Figure 4 Refining Process for Ulkenia DHA Oil



E. Chemical and Physical Characteristics of Ulkenia DHA Oil

The chemical and physical characteristics of Ulkenia DHA oil are presented in Table 2.

Table 2 Chemical and Physical Characteristics of Ulkenia DHA Oil

Characteristic	Description
Consistency	slightly waxy to liquid
Color	pale yellow to orange
pH value	7 (in ethanol)
Relative Density (25 °C)	0.95 g/mL

F. Specifications for Food-Grade Material

Ulkenia DHA oil is manufactured in accordance with current Good Manufacturing Practices (cGMP) and meets appropriate food-grade specifications. In order to ensure a consistent product, Lonza has established numerous physical, chemical, and heavy metal analyses for the final preparation. An analysis of 3 non-consecutive, representative lots of Ulkenia DHA oil (Table 3) demonstrates compliance with final product chemical and physical specifications.

Table 3 Chemical and Physical Specifications plus Analyses of Ulkenia DHA Oil

Test	Specification	Lot 990222	Lot 110708	Lot 120306
DHA [%] in the oil	38 to 50	38.1	42.0	38.2
Trans Fatty Acids (%)	≤ 1	< 1	< 1	< 1
Peroxide value [meq./kg]	≤ 5	1.8	0.5	3.6
Acid value [mgKOH/g]	≤ 0.5	0.08	0.03	0.03
Unsaponifiables [%]	≤ 4.5	0.3	0.5	0.4
Arsenic (ppm)	≤ 0.1	< 0.1	< 0.1	< 0.1
Lead (ppm)	≤ 0.1	< 0.1	< 0.1	< 0.1
Mercury (ppm)	≤ 0.1	< 0.004	< 0.004	< 0.004
Cadmium (ppm)	<0.1	< 0.1	< 0.1	< 0.1
Hexane (ppm)	< 1	< 1	< 1	< 1
Total viable counts (cfu[a]/g)	<1,000	<1,000	<1,000	<1,000
Coliforms (cfu/g)	<10	<10	<10	<10

[a] Colony forming units

The analysis of 3 non-consecutive, representative lots of Ulkenia DHA oil also provides compositional information with regard to the fatty acid composition, identification of the isolated triacylglycerol species, main triacylglycerol species, sterol content, main sterols, and trans fatty acids present in Ulkenia DHA oil. These data are presented in the Appendix (Tab 3).

4. Stability

Ulkenia DHA oil is sensitive to oxidative degradation on exposure to air, heat, and light, and should be used within 4 weeks after opening. The oil should be stored (also after opening) in tightly closed original packing in a cool (5 to 15°C) and dry place under inert atmosphere. Freezing will prolong the stability and at temperatures <5°C, Ulkenia DHA oil is stable for at least 24 months. The stability of Ulkenia DHA oil has been evaluated at 5 and 25°C. See Appendix (Tab 4) for details.

Ulkenia DHA oil has been evaluated at various storage temperatures. Changes in the primary oxidation products or DHA content (as % of total fatty acids) were not observed following refrigerated storage at +5°C and storage at room temperature (25°C) for up to 36 or 18 months, respectively. Data are shown in Appendix (Tab 4).

Stability in Food Applications

Stability investigations were also carried out in several food products and dietary supplement capsules containing Ulkenia DHA oil. In addition to chemical analyses, sensory analyses were carried out since traces of oxidation products of polyunsaturated fatty acids may impair the sensory quality of foods. Both chemical analyses and sensory studies demonstrated adequate stability.

Chemical analyses were carried out in soft gel capsules after storage at 25°C and 60% RH and 30°C and 70% RH. Recoveries were near complete and indicated a shelf-life without significant loss of at least 2 years. See (Table 5) in Appendix.

Analyses were also carried out in products of longer shelf-life, or exposed to high temperatures during processing. In a fruit juice, no loss was detected after 10 months of storage. In an energy bar losses of only 5 to 10% of the initial value were found after 8 to 16 months. In energy bars stored for 16 months and fresh cookies, the large majority of tasters did not find sensory deviations, while in stored cookies the general deterioration of sensory properties was similar to that for stored conventional cookies. Soft-gel capsules stored for approximately one and a half years after production were rated equivalent to commercial capsules based on fish oil.

III. SELF-LIMITING LEVELS OF USE

The use of Ulkenia DHA oil will be based on the maximum use levels of menhaden oil in specific food categories established by FDA for menhaden oils such that intake does not exceed 3.0 g/person/day. The use limitations of eicosapentaenoic acid (EPA) and DHA were based on the content of EPA and DHA within menhaden oil, which is approximately 20%., Therefore while Ulkenia DHA oil contains a DHA content of 38 to 50% and no significant EPA level, it can reasonably be concluded that approximately twice as much menhaden oil as Ulkenia DHA oil

will have to be consumed for the same intake of ω-3 fatty acids. Inversely, any limitation of use levels from Ulkenia DHA oil will therefore have to be less than 50% of the use levels of menhaden oil to ascertain compliance with the safe intake level. Consequently, the limitation of Ulkenia DHA oil use levels to 40% of the use levels in all food categories considered by the FDA for the GRAS use of menhaden oil would also ascertain compliance with the safe intake level of 3.0 g/person/day for the sum of EPA and DHA.

IV. BASIS FOR GRAS DETERMINATION

A. Documentation to Support the Safety of Ulkenia DHA Oil

The determination that Ulkenia DHA oil is GRAS is on the basis of scientific procedures, and the information supporting the general recognition of the safe use includes:

- published scientific data on the background consumption of DHA;
- safety of the source organism;
- preclinical and human clinical studies with *Ulkenia* derived DHA oil;
- the entirety of preclinical and human studies assessing the safety and nutritional value of DHA itself and DHA rich oils;
- the historical consumption of DHA from fatty fish (*e.g.*, haddock, tuna, salmon, mackerel) and other food sources;
- data pertaining to the identity, intended use, and estimated intake of DHA.

Moreover, these data were reviewed by a panel of experts, qualified by scientific training and experience to evaluate the safety of Ulkenia DHA oil as a component of food, who concluded that the proposed uses of Ulkenia DHA oil are safe and suitable and would be GRAS based on scientific procedures [see Appendix, Tab 1, entitled, " **EXPERT PANEL CONSENSUS STATEMENT: THE GENERALLY RECOGNIZED AS SAFE (GRAS) STATUS OF ULKENIA DHA OIL UNDER THE CONDITIONS OF INTENDED USE IN TRADITIONAL FOODS**. A summary of these data is presented herein.

B. Estimated Intake of Ulkenia DHA Oil

As mentioned, Ulkenia DHA oil is intended for use in a variety of food products. The proposed food uses and use levels are summarized in Table 4. The intended intake of DHA through the intended food uses of Ulkenia DHA oil will not exceed the limits assigned by the FDA through the menhaden oil GRAS, and is consistent with other ω-3, DHA-containing oils that have been notified to the FDA as being GRAS for similar uses (U.S. FDA, 2002a,b, 2004a,c). Since the DHA level in Ulkenia DHA oil is approximately 2 to 2.5 times higher than the combined levels of DHA and EPA in menhaden oil, the proposed use levels for Ulkenia DHA oil of 40% of those GRAS-affirmed for menhaden oil will ensure an intake within the limits considered safe by the

FDA. To ensure the safe use of the substance, the DHA rich oil will not be used in combination with any other oil that is a significant source of EPA or DHA.

Table 4 Intended Use and Use Levels of Ulkenia DHA Oil in Food[a]

Food Category	Intended Use Level (% w/w)
Baked goods, baking mixes (1)	2.0
Cereals (4)	1.6
Cheese products (5)	2.0
Chewing gum (6)	1.2
Condiments (8)	2.0
Confections, frostings (9)	2.0
Dairy product analogs (10)	2.0
Egg products (11)	2.0
Fats, oils (12) not in infant formula	4.8
Fish products (13)	2.0
Frozen dairy desserts (20)	2.0
Gelatins, puddings (22)	0.4
Gravies, sauces (24)	2.0
Hard candy (25)	4.0
Jams, jellies (28)	2.8
Milk products (31)	2.0
Nonalcoholic beverages (3)	0.2
Nut products (32)	2.0
Pastas (23)	0.8
Plant protein products (33)	2.0
Processed fruit juices (35)	0.4
Processed vegetable juices (36)	0.4
Snack foods (37)	2.0
Soft candy (38)	1.6
Soup mixes (40)	1.2
Sugar substitutes (42)	4.0
Sweet sauces, toppings, syrups (43)	2.0
White granulated sugar (41)	1.6

[a] Based on 21 CFR §184.1472 (U.S. FDA, 2008) and final rule 70 FR 14530, March 23, 2005.
The number in parenthesis following each food category refers to the paragraph listing of the particular food category in 21 CFR 170.3(n) (U.S. FDA, 2008).

C. Metabolic Fate of Ulkenia DHA Oil

Available evidence indicates that the absorption, distribution, and metabolism of DHA are similar to other dietary fatty acids. DHA is present in Ulkenia DHA oil in triglyceride form. Following enzymatic hydrolysis of the triglyceride in the upper intestine, the free fatty acid and

2-monoglycerides are incorporated into bile acid micelles for diffusion into the enterocytes, where they are incorporated into new triglycerides. Reconstituted triglycerides enter the lymph in the form of chylomicrons and are transported to the blood for distribution and may rapidly be incorporated into plasma lipid fractions, erythrocyte membranes, platelets, and adipose tissue. In passing through the capillaries of adipose tissue and liver, lipoproteins hydrolyze the chylomicron-contained triglycerides and phospholipids, with subsequent release of free fatty acids to the tissues for metabolism or reesterification into triglycerides and phospholipids for storage as energy. Generally, free fatty acids are transported across the mitochondrial membrane in the form of acyl-carnitine, where they then undergo β-oxidation with removal of 2 carbons from the fatty acid chain and production of acetic acid, a shorter-chained fatty acid, and acetyl CoA, which combines with oxaloacetic acid and enters the citric acid cycle for energy production. As fatty acids of 20 carbons or more are not easily transported across the mitochondrial membrane, DHA also may be metabolized *via* peroxisomal β-oxidation. In addition, it has been demonstrated, that DHA could be retroconverted to EPA (Geppert *et al.* 2005), which involves β-oxidation, auxiliary enzymes, and removal of 2 carbon units from the carboxyl end of the fatty acid.

D. Preclinical Studies Pertaining to the Safe Consumption of Ulkenia DHA Oil

The safety of Ulkenia DHA oil is supported by a number of toxicological studies conducted in mice and rats, specifically acute, subchronic and single generation reproduction studies, as well as mutagenicity studies conducted using *Salmonella typhimurium*, *Escherichia coli* WP2 *uvrA* (with or without metabolic activation), and a chromosomal aberration assay in Chinese hamster fibroblast cells (Fujii and Suwa, 1998a; Kashima and Sarwar, 2000; Neda, 2000a; Bruijntjes-Rozier and van Ommen, 2001; Kuilman and Waalkens-Berendsen, 2001; Kroes *et al.*, 2003; Blum *et al.*, 2007a,b). These data support the safety of Ulkenia DHA oil under the intended conditions of use. Summaries of these studies are presented below.

All studies were conducted in accordance with the OECD Principles of Good Laboratory Practice (GLP) [Organisation for Economic Cooperation and Development, OECD Principles of Good Laboratory Practice (as revised in 1997)], Paris. ENV/MC/CHEM(98)17 (OECD, 1998a).

- OECD Guideline no. 471, Genetic Toxicology: Bacterial Reverse Mutation Test, adopted 21 July 1997 (OECD, 1997).

- OECD Guideline for Testing of Chemicals no. 415, One-Generation Reproduction Toxicity Study (adopted 26 May 1983) (OECD, 1983).

- OECD Guideline for Testing of Chemicals no. 401, Acute Oral Toxicity, 1987 (OECD, 1987).

- OECD Guideline for Testing of Chemicals no. 408, Repeated Dose 90-day Oral Toxicity Study in Rodents, 1998 (OECD, 1998b).

1 Acute Toxicity Studies

A single oral dose of Ulkenia DHA oil (reported as DHA45-oil) (Kroes *et al.*, 2003) administered to 5 ICR male mice and 10 Sprague-Dawley [Crj/CD(SD)IGS] rats (5 rats/sex) at a dose of 2 g/kg body weight was reported not to produce clinical signs of toxicity during a 14-day post-dosing observation period or gross or histopathological compound-related changes at necropsy (Fujii and Suwa, 1998b; Neda, 2000b). Watery diarrhea was reported in 2 male rats at 6 hours following administration of the test compound. Oral LD_{50} values of greater than 2,000 mg Ulkenia DHA oil/kg body weight were reported for both studies.

2 Subchronic Toxicity Studies

In a 90-day study, groups of 15 male and 15 female rats were orally administered water or Ulkenia DHA oil at concentrations of 0, 500, 1,000, and 2,000 mg/kg in combination with 2,000, 1,500, 1,000, or 0 mg/kg DHA-containing fish oil, respectively. Additional animals were administered water, 2,000 mg/kg Ulkenia DHA oil, or 2,000 mg/kg fish oil for 90 days, followed by a 4-week recovery phase. No compound-related effects were observed in clinical observations, food and water consumption, mortality, gross pathology and histopathology. Increased body weights and liver weights in oil-treated groups were attributed to the large lipid load and were not regarded as toxicologically significant. Furthermore, no treatment-related differences in the measured parameters between the Ulkenia DHA oil and fish oil groups were detected (Blum *et al.*, 2007a).

3 Reproductive Toxicity Studies

The reproductive toxicity of Ulkenia DHA oil was assessed in a one-generation study. Groups of 28 male and 28 female rats were administered powdered diets containing Ulkenia DHA oil at concentrations of 1.5, 3.0, or 7.5%, and the control group received a diet containing 7.5% corn oil. Males and females were treated for 10 weeks prior to mating and during mating. Females continued to receive test diets during gestation and lactation. In parental animals, clinical observations, mortality, fertility and reproductive performance (precoital time, mating index, fertility indices, fecundity, gestation index and duration, numbers of stillborn pups and post-implantation losses) were unaffected by treatment. Differences in food consumption, body weight, and liver weight in the treated groups were not considered to be due to an adverse effect of Ulkenia DHA oil. Spleen weight increases in treated animals were associated with extramedullary hematopoiesis. Yellow discoloration of abdominal adipose tissue was observed in rats from the high-dose group, and histological examination revealed steatitis in all treated parental groups. Exposure to Ulkenia DHA oil did not influence the physical development of F1 animals. There were no adverse effects of treatment on the numbers of pups, pups/litter, pup mortality, sex ratio, or pup weight. There was no evidence of a compound-related effect on the incidence of malformations. These results demonstrate that Ulkenia DHA oil at dietary

concentrations of up to 7.5% in rats does not affect reproductive capacity or pup development (Blum *et al.*, 2007b).

Mutagenicity/Genotoxicity and Carcinogenicity Studies

Ulkenia DHA oil derived from *Ulkenia* sp. (reported as DHA45-oil) was reported to produce negative results in the Ames assay (up to 5 mg per plate) using *S. typhimurium* strains TA97, TA98, TA100, TA102, TA1535, and TA1537 and *E. coli* WP2 *uvrA* with or without metabolic activation (Fujii and Suwa, 1998a; Bruijntjes-Rozier and van Ommen, 2001). Additionally, Ulkenia DHA oil was reported not to produce chromosomal aberrations in Chinese hamster fibroblast cells with or without metabolic activation (Kashima and Sarwar, 2000; Blum *et al.*, 2007a).

E. Studies in Humans

Largely, the available clinical data in humans do not provide evidence that DHA, at estimated exposures provided through the use of Ulkenia DHA oil, would have an adverse effect on low-density lipoprotein (LDL)-cholesterol levels, glycemic control, bleeding time, platelet aggregation, or other hemostatic parameters. However, some effects on low-density lipoprotein LDL-cholesterol have been reported. Geppert *et al.* (2005, 2006) evaluated the effects of Ulkenia DHA oil from microalgae *Ulkenia* sp. on plasma lipids and several safety parameters in a double-blind, placebo-controlled, parallel design intervention study. Normolipedemic vegetarians (87 females, 27 males) consumed 2.28 g Ulkenia DHA oil (0.94 g DHA/day) or olive oil for 8 weeks. Intake of DHA markedly increased the DHA content of plasma and red blood cell phospholipids. Absolute and relative changes in the omega-3 index were inversely correlated to baseline levels, with the highest increases seen in subjects with the lowest levels at baseline. DHA supplementation decreased plasma triglycerol levels by 23%. Plasma total, LDL, and high-density lipoprotein (HDL) cholesterol significantly increased in the DHA group, resulting in lower triglyceride (TG):HDL cholesterol and unchanged LDL:HDL and total cholesterol:HDL cholesterol ratios. No physiologically relevant changes in safety, hemostatic factors, or liver or cardiac enzymes were noted, indicating that the Ulkenia DHA oil from *Ulkenia* sp was well tolerated. Reported side effects were equally distributed between the DHA and placebo groups. Although DHA improved some risk factors for coronary heart disease (plasma TG, TG: HDL cholesterol), LDL cholesterol also increased.

Similarly, the data do not indicate that DHA would produce adverse effects on immune function or response, kidney or liver function, or lipid peroxidation. Moreover, following a thorough evaluation of several published human studies, the FDA affirmed that menhaden oil, partially hydrogenated menhaden oil, and fully hydrogenated menhaden oil are GRAS under the conditions of intended use as direct human food ingredients, and concluded that consumption of up to 3 g DHA + EPA combined/person/day does not pose a "significant risk for increased bleeding time", has no "clinically significant effect on glycemic control", and is "safe with respect

to the effect on LDL cholesterol" (U.S. FDA, 1989, 1997). Subsequent to the GRAS affirmation of menhaden oil in 1997, additional studies conducted to determine potential effects of DHA-containing oils on hemostatic parameters, glycemic control, and LDL-C were conducted in humans. Overall, the results of these studies indicate that DHA, provided in fish or marine-derived oils alone or in combination with DPA and/or EPA, at levels up to 6 g DHA/person/day, would not produce significant adverse effects on these parameters. These results are consistent with the earlier conclusions of the U.S. FDA (1997). Additionally, the FDA published a letter regarding the dietary supplement qualified health claim for ω-3 fatty acids and heart disease, stating that the use of EPA and DHA ω-3 fatty acids, including DHA from marine algae, as dietary supplements is safe (U.S. FDA, 2000). When viewed in its entirety, the scientific evidence indicates that the consumption of Ulkenia DHA oil, under the conditions of intended use, would not be expected to produce adverse effects on human health.

F. Other Data Pertaining to the Safety of Ulkenia DHA oil

1. Safety of Source Organism

The safety of Ulkenia DHA oil is further substantiated given that the source organism, *Ulkenia* sp., a thraustochytrid and a member of the Chromista kingdom (Bahnweg, 1979a; Cavalier-Smith *et al.*, 1994; Honda *et al.*, 1999; European Register of Marine Species, 2001), has not been reported to produce toxins or to demonstrate pathogenicity (OmegaTech GmbH, 1997; Van Dolah, 2001). Thraustochytrids are known to exhibit worldwide distribution, having been identified in plankton and other marine detritus, as well as comprising a portion of the diet of filter-feeding invertebrates in the marine ecosystem (Ulken *et al.*, 1990; Sathe-Pathak *et al.*, 1993; Azevedo and Corral, 1997; Naganuma *et al.*, 1998). Due to their wide distribution throughout the marine ecosystem, thraustochytrids constitute an indirect component of the human diet through the consumption of marine creatures (*e.g.*, clams or mussels).

The source organism has been demonstrated to be nonmutagenic in the Ames assay using Salmonella typhimurium strains TA97, TA98, TA100, and TA102 with or without metabolic activation (Fujii and Suwa, 1999). In a 14-day feeding study designed to determine the safety of *Ulkenia* sp., the microorganism was suspended in distilled water administered orally to ICR male mice (SPR) at a dose of 2 g *Ulkenia* sp./kg body weight/day (Celanese Ventures, 1999). A control group received distilled water only. No significant differences in clinical signs, body weight gains, or necropsy observations were reported.

Additional information pertaining to the taxonomical classification and microalgal toxin analysis is provided in the Appendix (Tab 2).

2. Safety of Palmitic Acid

The total fatty acid content of Ulkenia DHA oil comprises approximately 33% palmitic acid (16:0) of total fatty acids. Palmitic acid is a natural component of the diet and occurs mainly in meat, poultry, fish, grain products, and milk and milk products (Jonnalagadda *et al.*, 1995). As part of an analysis of the fatty acid intake pattern and the contribution of different food groups to the fatty acid intake of the American population, Jonnalagadda *et al.* (1995) reported the consumption of palmitic acid in the U.S. to range from 11 to 19 g/day, or 0.18 to 0.32 g/kg body weight/day for the average 60 kg individual.

The exposure to DHA from the intended food uses will be a maximum of 3 g/day under the conditions of intended use. On the basis of Ulkenia DHA oil containing a minimum of 43% DHA of the total fatty acid content, the maximum intake of palmitic acid corresponds to approximately 1 g/day. This intake is at least 10 times less than the amount of palmitic acid already consumed as a natural constituent of the diet, and thus the estimated intake of palmitic acid through the intended use of Ulkenia DHA oil would not have an impact on the relative amount of daily dietary palmitic acid. Therefore, the estimated intake of palmitic acid through the proposed uses of Ulkenia DHA oil would not be expected to produce adverse effects on human health.

3. Safety of Docosapentaenoic Acid (DPA)

Analysis of the fatty acid component of Ulkenia DHA oil revealed the presence of 2 forms of DPA (22:5): ω-6 DPA (8 to 14% total fatty acids), and ω-3 DPA (0.15 to 0.6% total fatty acids). Both ω-6 DPA and ω-3 DPA have been reported to occur naturally in the phospholipid membranes of tissues and organs, such as endothelial cells, retina, testes, and liver (Chanmugam *et al.*, 1984; Milks and Sprecher, 1985; Alvarez *et al.*, 1994; Achard *et al.*, 1995; Kanayasu-Toyoda *et al.*, 1996). Additionally, both DPA isomers have been identified in fish, seal, and microalgal-derived oils, with the ω-3 isomer being more abundant in fish and seal oils (Haglund *et al.*, 1998; Akiba *et al.*, 2000; Tam *et al.*, 2000).

As both DPA isomers are component acids of fish oil, the safety of ω-3 and ω-6 DPA is supported by the numerous non-clinical and clinical studies that have reported no adverse effects following dietary supplementation with fish or marine oil. Analysis of 3 lots of Ulkenia DHA oil indicates concentrations of ω-3 and ω-6 DPA of approximately 0.3 and 12%, respectively of the total fatty acids (Section 3.4). At these levels, the estimated intake of DPA (ω-3 + ω-6) through the intended conditions of use of Ulkenia DHA oil would amount to a maximum of <1 g/person/day. This intake is within the range of levels of DPA provided via fish oil supplementation in clinical studies that were reported not to produce adverse effects. Therefore, the estimated intake of ω-3 and ω-6 DPA through the proposed uses of Ulkenia DHA oil would not be expected to produce adverse effects in humans.

4. Safety of Sterols

The safety of dietary cholesterol and phytosterols is well documented in the scientific literature. Cholesterol and phytosterols are natural constituents of the diet and 4-methyl sterols occur in the endogenous metabolic pathway of cholesterol biosynthesis. These intakes are well below the amounts of sterols already consumed as natural constituents in the diet (up to 400 mg/person/day), and thus, the estimated intake of sterols through the proposed uses of Ulkenia DHA oil would not have an impact on the relative amount of cholesterol and phytosterols already consumed in the diet. Therefore, the dietary exposure to cholesterol, 7-dehydrostigmasterol, and 4-methyl-chondrillasterol from the intended use of would not be expected to produce adverse effects on human health. The safety of the individual sterol components present in Ulkenia DHA oil has also been confirmed through the various safety studies conducted on Ulkenia DHA oil itself which showed no adverse effects.

5. Safety Data for Other DHA-Containing Oils

The safety of DHA-containing fish and microalgal-derived oils has been studied extensively in toxicological studies, and due to their similarity in composition, these studies further support the safety of Ulkenia DHA oil. Overall, the data from these studies indicate that gavage or dietary administration of fish or microalgal-derived oils does not produce significant adverse effects on mortality, body weight gains, food consumption, or clinical observations in laboratory animals including mice, rats, and pigs (Danse and Verschuren, 1978a; Ruiter *et al.*, 1978; Willumsen *et al.*, 1993; Boswell *et al.*, 1996; Hempenius *et al.*, 1997, 2000; Wibert *et al.*, 1997; Rabbani *et al.*, 1999; Arterburn *et al.*, 2000a,b; Oarada *et al.*, 2000; Hammond *et al.*, 2001a,b,c). Yellow fat disease, which is characterized by accretion of lipofuscin pigment, degeneration of adipose cells (steatosis), and inflammation of adipose tissue (steatitis) (Danse and Steenbergen-Botterweg, 1976; Danse and Verschuren, 1978a; Danse *et al.*, 1979), has been reported to occur naturally in wildlife species and was reported in rats, rabbits, mink, and pigs in various toxicological studies following the consumption of diets rich in ω-3 PUFAs (*i.e.*, ω-3 PUFA-containing oils at dietary concentrations ranging from as high as 12 to 19% for periods of 8 weeks to 12 months in length) in combination with a vitamin E deficient state (Jones *et al.*, 1969; Helgebostad and Ender, 1973; Danse and Steenbergen-Botterweg, 1976, 1978; Danse and Verschuren, 1978a,b; Ruiter *et al.*, 1978; Danse *et al.*, 1979; Charnock *et al.*, 1987; Verschuren *et al.*, 1990; Farwer *et al.*, 1994; Hempenius *et al.*, 1997, 2000). This effect is considered a normal response to a large load of dietary lipids, and may be prevented by concurrently administering vitamin E with high-PUFA diets (Helgebostad and Ender, 1973; Verschuren *et al.*, 1990; Farwer *et al.*, 1994; Muggli, 1994; Ando *et al.*, 2000). Some study authors reported increased liver and spleen weights in mice and rats administered high PUFA diets at daily intakes ranging from 25 to 9,500 mg/kg body weight and varying durations of 4 to 13 weeks (Danse and Verschuren, 1978a,b; Boswell *et al.*, 1996; Hempenius *et al.*, 1997; McGuire *et al.*, 1997; Burns *et al.*, 1999a; Rabbani *et al.*, 1999; Oarada *et al.*, 2000); however, no histopathological effects were observed in these

organs and the increased organ weights were reported to be adaptations to accommodate the large lipid load.

The safety of Ulkenia DHA oil is further corroborated by an extensive number of human clinical studies with endpoints relevant to determining the safety and metabolism of DHA (von Schacky *et al.*, 1985; Hostmark *et al.*, 1988; Mehta *et al.*, 1988; Radack *et al.*, 1989; Bønaa *et al.*, 1992; Deslypere, 1992; Haglund *et al.*, 1992, 1998; Kenny *et al.*, 1992; Mori *et al.*, 1992, 1997, 2000; Saynor and Gillott, 1992; Schmidt *et al.*, 1992; Almdahl *et al.*, 1993; Clark *et al.*, 1993; Connor *et al.*, 1993; Hansen *et al.*, 1993a,b, 1998a,b; Harris *et al.*, 1993, 1997; Krokan *et al.*, 1993; Mundal *et al.*, 1993; Nilsen *et al.*, 1993; Swails *et al.*, 1993; Axelrod *et al.*, 1994; Chin and Dart, 1994; Cirillo *et al.*, 1994; de Maat *et al.*, 1994; Eritsland *et al.*, 1994a,b, 1995a,b, 1996; Henderson *et al.*, 1994; Leaf *et al.*, 1994; Lungershausen *et al.*, 1994; Mackness *et al.*, 1994; McVeigh *et al.*, 1994; Mundal *et al.*, 1994; Nordøy *et al.*, 1994, 1998; Prisco *et al.*, 1994, 1995; Turini *et al.*, 1994; Bonnema *et al.*, 1995; Christensen *et al.*, 1995; Luostarinen *et al.*, 1995; Misso and Thompson, 1995; Morgan *et al.*, 1995; Sacks *et al.*, 1995; Toft *et al.*, 1995, 1997; Tremoli *et al.*, 1995; Ågren *et al.*, 1996, 1997; Bagdade *et al.*, 1996; Balestrieri *et al.*, 1996; Conquer and Holub, 1996, 1998; Engström *et al.*, 1996; Fasching *et al.*, 1996; Gray *et al.*, 1996; Hamazaki *et al.*, 1996; Innis and Hansen, 1996; Lenzi *et al.*, 1996; McGrath *et al.*, 1996; McManus *et al.*, 1996; Otto *et al.*, 1996; Palozza *et al.*, 1996; Rivellese *et al.*, 1996; Rossing *et al.*, 1996; Silva *et al.*, 1996; Wigmore *et al.*, 1996; Yosefy *et al.*, 1996; Adler and Holub, 1997; Badalamenti *et al.*, 1997; Brister and Buchanan, 1998; Freese and Mutanen, 1997a,b; Goh *et al.*, 1997; Grimsgaard *et al.*, 1997, 1998; Nelson *et al.*, 1997; Roulet *et al.*, 1997; Sanders *et al.*, 1997; Tsai and Lu, 1997; Gogos *et al.*, 1998; Kelley *et al.*, 1998, 1999; Swahn *et al.*, 1998; Burns *et al.*, 1999b; Conquer *et al.*, 1999; Grundt *et al.*, 1999; Pirich *et al.*, 1999; Seljeflot *et al.*, 1999; Véricel *et al.*, 1999; Wensing *et al.*, 1999; Almallah *et al.*, 2000; Wallace *et al.*, 2000; Yaqoob *et al.*, 2000; Woodman *et al.*, 2003). As reviewed by Kroes *et al.* (2003) and as reported by the Expert Panel, human studies include metabolic studies indicating that the absorption, distribution, and metabolism of DHA is similar to other fatty acids, as well as, studies examining the effect of DHA-containing oils on bleeding times and other hemostatic parameters, glycemic control, LDL cholesterol and other plasma lipid fractions, as well as, additional measured endpoints (*e.g.*, immune function, liver and kidney function) related to the safety of DHA. Overall, the results of these studies provide evidence that DHA, at estimated exposures provided through the intended use of Ulkenia DHA oil, would not have an adverse effect on human health. The available data further indicates other components of Ulkenia DHA oil, namely palmitic acid, docosapentaenoic acid, and sterols, would not have adverse effects on human health (Kroes *et al.*, 2003) under the intended conditions of use of Ulkenia DHA oil.

The consumption of DHA-containing oils providing up to 6 g DHA/day, alone or in combination with DPA and/or EPA, has been reported to result in alterations of platelet and total serum phospholipid and nonesterified fatty acid compositions, with increases in the levels of ω-3 PUFAs, including DHA, and concomitant decreases in levels of arachidonic acid (AA) (20:4;

ω-6) of up to 26% (Mori *et al.*, 1992, 1997; Schmidt *et al.*, 1992; Mundal *et al.*, 1993; Eritsland *et al.*, 1994a; Henderson *et al.*, 1994; Leaf *et al.*, 1994; Turini *et al.*, 1994; Luostarinen *et al.*, 1995; Tremoli *et al.*, 1995; Conquer and Holub, 1996; Engström *et al.*, 1996; Nelson *et al.*, 1997; Conquer and Holub, 1998; Haglund *et al.*, 1998; Conquer *et al.*, 1999; Yaqoob *et al.*, 2000). Arachidonic acid, either obtained in the diet or as a biosynthetic product of linoleic acid (18:2; ω-6), occurs as a fatty acid component of platelet membranes and most tissue phospholipids functioning as the main precursor of eicosanoids, which are involved in mediation of hemostatic parameters and immune cell function and response (Linder, 1991; Foegh *et al.*, 1998; Kelley and Rudolph, 2000). Critical review of the scientific literature indicates that while AA levels may decrease following consumption of ω-3 PUFAs, the concentration of AA generally remains within normal physiological concentrations of 5 to 15% of total fatty acids of platelet membranes and tissue phospholipids (Kroes *et al.*, 2003). There is no indication that DHA, at exposures estimated through the proposed uses of Ulkenia DHA oil, would adversely affect hemostatic parameters or immune function or response as a result of possible concomitant decreases in platelet and total serum phospholipid and nonesterified fatty acid levels of AA (Haglund *et al.*, 1992, 1998; Mori *et al.*, 1992, 1997; Schmidt *et al.*, 1992; Eritsland *et al.*, 1994a; Henderson *et al.*, 1994; Leaf *et al.*, 1994; Parkinson *et al.*, 1994; Turini *et al.*, 1994; Luostarinen *et al.*, 1995; Tremoli *et al.*, 1995; Conquer and Holub, 1996; Engström *et al.*, 1996; Nelson *et al.*, 1997; Gogos *et al.*, 1998; Kelley *et al.*, 1998, 1999; Conquer *et al.*, 1999; Almallah *et al.*, 2000; Yaqoob *et al.*, 2000).

6. GRAS Status of Related Products

Due to the compositional similarity and DHA content of fish and marine algal-derived oils to Ulkenia DHA oil, the available scientific literature on the safety of these oils supports the safety of Ulkenia DHA oil.

In 1989, the FDA affirmed the GRAS status of partially hydrogenated menhaden oil (with an iodine number ≤85) and fully hydrogenated menhaden oil for use in foods with certain limitations (U.S. FDA, 1989). Subsequently, in 1997, the FDA affirmed the GRAS status of menhaden oil and partially hydrogenated menhaden oil (with an iodine number ≤110), provided that under the conditions of intended use in foods, the total EPA + DHA daily intake does not exceed 3 g/person/day (U.S. FDA, 1997). In 2005, FDA issued a final rule on menhaden oil reallocating the use levels and categories of use within the GRAS affirmation, but ensuring daily intakes of EPA and DHA do not exceed 3 g/person/day (U.S. FDA, 2005).

The FDA has responded that it had no questions concerning the notifier's GRAS determination for DHASCO, a DHA-containing oil derived from the algae *Crypthecodinium cohnii* in infant formulas (GRN 000041 – U.S. FDA, 2001) and an algal oil, derived from the thraustochytrid species *Schizochytrium* sp., containing approximately 35% DHA (DHA Gold, now DHASCO-S; GRN 000137 – U.S. FDA, 2004a), with intended uses as a direct food ingredient in the same categories as considered GRAS for menhaden oil (U.S. FDA, 2004a).

Further DHA containing oil products based on fish oils with GRAS status:

Ocean Nutrition Canada: GRN 000138 – U.S. FDA, 2004b.

Twin Rivers Technologies: 55% DHA/EPA from fish oil: GRN 000200 – U.S. FDA, 2006a,

Puleva Biotech GRN 000193 – U.S. FDA, 2006b

Unilever, Marinol Omega-3: GRN 000105 – U.S. FDA, 2002a

Jedwards International: GRN 000102 – U.S. FDA, 2002c

Tuna DHA plus ARA for infant formula. Ross Products Division, Abbott Laboratories (Ross) GRN 000094 – U.S. FDA, 2006c

G. Summary and Basis for GRAS Conclusion

In humans, DHA is a critical component of most cell membranes and tissues. DHA is formed *in vivo* from the essential fatty acid, α-linolenic acid, by the action of elongase and desaturase enzymes, or is consumed in the diet, primarily in fish-based foods. Available scientific evidence indicates that the absorption, distribution, and metabolism of dietary DHA are similar to other dietary fatty acids.

The safety of DHA is well established in the literature based on the historical consumption of fish, and fish- and marine-based products. In 1997, the FDA affirmed that menhaden oil, providing up to 3 g/person/day total DHA + EPA, is GRAS under the conditions of intended use as a direct human food ingredient (U.S. FDA, 1997). FDA also accepted GRAS notification for 2 algal derived DHA rich oils, from *Schizochytrium* and *C. cohnii* (GRN 000041 – U.S. FDA, 2001 and GRN 000137 – U.S. FDA, 2004a) and also various fish derived DHA containing oil products: GRN 000102 – U.S. FDA, 2002c, GRN 000105 – U.S. FDA, 2002a, GRN 000094 – U.S. FDA, 2006c, GRN 000193 – U.S. FDA, 2006b, GRN 000200 – U.S. FDA, 2006a, GRN 000138 – U.S. FDA, 2004b.

Various studies in animal models, including rats and rabbits, indicate that DHA, as present in marine oils, does not produce adverse effects on mortality, body weight gains, food consumption, or clinical observations. Prospective clinical trials and epidemiological studies, as well as reported dietary intervention studies using levels of DHA that are similar to, or greater than, the estimated intake from the intended food uses of Ulkenia DHA oil, indicate that these levels are well-tolerated by humans, and are without reported adverse effects. In addition, there is wide range of published literature regarding the safety of DHA-containing oils derived from microalgal sources, including organisms related to *Ulkenia* sp.

All components of Ulkenia DHA oil have history of occurrence in the diet, and review of the existing scientific literature on the safety of DPA and sterols indicate that these components of Ulkenia DHA oil would not produce adverse effects on human health. While the microalgae, *Ulkenia* sp., is unique as a source of DHA for use as a food ingredient, *Ulkenia* sp. is considered both non-toxigenic and non-pathogenic to man.

The results of studies conducted on Ulkenia DHA oil (Kroes, 2003, Geppert *et al.*, 2005, 2006; Blum 2007a,b) as well as information identified in the literature have been determined by Lonza not to indicate any potential for adverse effects in humans following consumption of the Ulkenia DHA oil ingredient under the intended conditions of use. Ulkenia DHA oil is similar in composition to DHA-containing fish and microalgal oils, which have a history of consumption as part of a normal diet. The intended intake of DHA through the intended food uses of Ulkenia DHA oil will not exceed the limits assigned by the FDA through the menhaden oil GRAS, and is consistent with other ω-3, DHA-containing oils that have notified the FDA as GRAS for similar uses, both from fish oil and algal sources, see above (U.S. FDA, 2002a,b, 2004a,c). Therefore, Lonza has concluded that Ulkenia DHA oil is GRAS under the intended conditions of use on the basis of scientific procedures. This determination is further supported by an expert panel evaluation of the safety of Ulkenia DHA oil under the intended conditions of use.

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APPENDIX

APPENDIX

Ulkenia DHA Oil GRAS NOTIFICATION

Notifier: Lonza Ltd.
Muenchensteinerstrasse 38
CH-4002
Basel, CH-4002
Switzerland

TAB 1

EXPERT PANEL CONSENSUS STATEMENT REGARDING THE GENERALLY RECOGNIZED AS SAFE (GRAS) STATUS OF ULKENIA DHA OIL FOR USE IN FOODS

(TAB 1)

EXPERT PANEL CONSENSUS STATEMENT: THE GENERALLY RECOGNIZED AS SAFE (GRAS) STATUS OF ULKENIA DHA OIL UNDER THE CONDITIONS OF INTENDED USE IN TRADITIONAL FOODS

November 2, 2009

INTRODUCTION

At the request of Lonza Ltd (Lonza), an Expert Panel (the "Panel") of independent scientists, qualified by their relevant national and international experience and scientific training to evaluate the safety of food ingredients, was specially convened to conduct a critical and comprehensive evaluation of the available pertinent data and information, and determine whether, under the conditions of intended use as a nutrient in traditional foods, Ulkenia DHA oil would be "generally recognized as safe" (GRAS), based on scientific procedures. Ulkenia DHA oil is a docosahexaenoic acid (DHA)-rich triacylglycerol oil derived from *Ulkenia sp.* microalgae by Lonza. The Panel consisted of the below-signed qualified scientific experts: Robert Kroes, D.V.M., Ph.D. (Utrecht University deceased), Ernst Schaefer, M.D. (Tufts University), and Gary Williams, M.D. (New York Medical College).

The Panel, independently and collectively, critically evaluated a comprehensive package of scientific safety information and data compiled from the literature and other published sources initially through September 2004. The Panel also evaluated any other data and information, published or unpublished, that the Panel deemed appropriate or pertinent to safety of Ulkenia DHA oil under the conditions of intended use. In addition, the Panel considered data and information provided by Lonza, including information pertaining to the method of manufacture and product specifications, analytical data, intended use levels in specified food products, consumption estimates for all intended uses, data on the safety of similar DHA-containing fish and marine oils, and unpublished corroborating toxicological studies conducted on Ulkenia DHA oil and the source microalgae. Since September 2004 no further scientific information has been published that would alter the opinion of the remaining Panel members regarding the original safety assessment of Ulkenia DHA oil under the proposed conditions of intended use.

Following independent, critical evaluation of such data and information, the Panel unanimously concludes that under the conditions of intended use in traditional foods described herein, Ulkenia DHA oil, meeting appropriate food-grade specifications, and manufactured and used in accordance with current good manufacturing practice, is GRAS based on scientific procedures. A summary of the basis for the Panel's conclusion, excluding confidential data and information, is provided below.

MANUFACTURING AND COMPOSITION

Ulkenia DHA oil is produced in accordance with current good manufacturing practice following multi-step fermentation of a genetically stable and pure culture of the marine protist, *Ulkenia sp.* Ulkenia DHA oil is extracted from the microalgae *via* a solvent-based extraction process using food grade solvents and processing aids, and is further refined by processes standard to the edible oil industry, including degumming, deacidification, bleaching and deodorization, using materials appropriate for food processing. Specifications for Ulkenia DHA oil are presented in Table 1. Analysis of nonconsecutive representative lots demonstrated compliance with final product chemical, physical, and microbiological specifications, and stability under recommended storage conditions.

Table 1 Product Specifications for Ulkenia DHA oil

Parameter	Specification
DHA	38 to 50 (% by weight)
Trans Fatty Acids	≤ 2 %
Peroxide value	≤ 5 meq./kg
Acid value	≤ 0.5 mg KOH/g
Moisture and Volatiles	≤ 0.05 %
Unsaponifiables	≤ 4.5 %
Lead	≤ 0.1 ppm
Arsenic	≤ 0.5 ppm
Mercury	≤ 0.5 ppm

Ulkenia DHA oil is a refined, food grade oil of >95% triacylglycerols, with a total fatty acid composition of 38 to 50% DHA. The remaining fatty acids of Ulkenia DHA oil are comprised mainly of saturated palmitic acid (16:0) (28 to 37%) and a lesser amount (8 to 14%) of the ω-6 fatty acid, docosapentaenoic acid (DPA) (22:5). In addition, the Ulkenia DHA oil product will contain safe and suitable antioxidants permitted by the FDA for use in edible oils to ensure stability.

INTENDED USE AND ESTIMATED INTAKE

The main dietary source of polyunsaturated fatty acids (PUFAs), such as DHA, is fish, particularly fatty fish, in which concentrations of total ω-3 fatty acids range from 0.1 to 5.3 g/ 100 g (1, 2). In the U.S., the usual consumption of ω-3 fatty acids amounts to approximately 1,600 mg/day, predominantly as α-linolenic acid (18:3), but also including eicosapentaenoic acid (EPA), DHA, and DPA. Of the total amount of ω-3 fatty acids consumed per day in the U.S., an estimated 100 to 200 mg is consumed as DHA (3, 4). Other populations with a traditionally

higher consumption of DHA-containing foods, such as Japan, Norway, South Africa, and the Portuguese island of Madeira, have average DHA intakes of approximately 500 to 700 mg DHA/day (5-9).

Several international scientific authorities have published recommendations for daily intakes of ω-3 PUFAs. For example, although the nutrition recommendations of Health Canada do not distinguish the identities of individual ω-3 PUFAs, they provide a recommended daily intake of 1,000 to 1,800 mg ω-3 PUFAs/day, in a ratio of 1:4 to 1:10 with ω-6 PUFAs (10). A workshop convened by the International Society for the Study of Fatty Acids and Lipids (ISSFAL) concluded there was insufficient data to determine a Dietary Reference Intake (DRI) for ω-3 PUFAs; however, Adequate Intakes (AIs) of a minimum 220 mg/day each for DHA and EPA, and 650 mg/day for DHA and EPA combined were recommended (11). In Europe, the British Nutrition Foundation has recommended a desirable population intake of 1,100 mg (females) and 1,400 mg (males) of DHA and EPA/day (12).

Several ω-3 fatty acids, such as EPA (20:5) and DHA (including DHA from marine algae) also are sold as dietary supplements, and the U.S. Food and Drug Administration (FDA) currently allows a qualified health claim for ω-3 fatty acids and a reduced risk of coronary heart disease to appear in labeling for dietary supplement products (13). Additionally, under certain conditions of intended use in foods, similar oils, such as fully hydrogenated, partially hydrogenated, and refined menhaden oils, are affirmed as GRAS by FDA for use in several specified foods (21 CFR §184.1472) (14, 15). The recognized difference between menhaden oil, a refined oil derived from menhaden fish, and edible vegetable oils and animal fats is the higher percentage of PUFAs with 4 to 6 double bonds in menhaden oil, including EPA (13.7%), DHA (6.6%), and DPA (1.9%) (15). Furthermore, other DHA-containing oil ingredients are used in a number of traditional food products, as the FDA has responded with no objections against several GRAS notices submitted for other sources of ω-3 fatty acids including some for uses identical to those specified as GRAS for menhaden oil (16, 17, 206, 207, 208, 209, 2010, 2011, 2012).

The only notable differentiation regarding Ulkenia DHA oil as compared to other DHA-containing products already on the market is the production source. Ulkenia DHA oil is derived from a fermentation process using a nontoxigenic and nonpathogenic marine protist, which has not previously been used in the production of food products or ingredients; never the less, the fatty acids identified in Ulkenia DHA oil are the same as those of fish and microalgal-derived oils already consumed by the human population.

Lonza intends to market Ulkenia DHA oil as a food ingredient to increase the intake of dietary ω-3 PUFAs, particularly DHA, a critical component of most cell membranes and tissues.

Ulkenia DHA oil is intended for use in a variety of food products. The proposed food uses and use levels are summarized in Table 2. The intended intake of DHA through the intended food uses of Ulkenia DHA oil will not exceed the limits assigned by the FDA through the menhaden

oil GRAS, and is consistent with other ω-3, DHA-containing oils that have been notified to the FDA as being GRAS for similar uses (U.S. FDA, 2002a,b, 2004a,c). Since the DHA level in Ulkenia DHA oil is approximately 2 to 2.5 times higher than the combined levels of DHA and EPA in menhaden oil, the proposed use levels for Ulkenia DHA oil of 40% of those GRAS-affirmed for menhaden oil will ensure an intake within the limits considered safe by the FDA. To ensure the safe use of the substance, the DHA rich oil will not be used in combination with any other oil that is a significant source of EPA or DHA.

Table 2 Intended Use and Use Levels of Ulkenia DHA Oil in Food[a]

Food Category	Intended Use Level (% w/w)
Baked goods, baking mixes (1)	2.0
Cereals (4)	1.6
Cheese products (5)	2.0
Chewing gum (6)	1.2
Condiments (8)	2.0
Confections, frostings (9)	2.0
Dairy product analogs (10)	2.0
Egg products (11)	2.0
Fats, oils (12) not in infant formula	4.8
Fish products (13)	2.0
Frozen dairy desserts (20)	2.0
Gelatins, puddings (22)	0.4
Gravies, sauces (24)	2.0
Hard candy (25)	4.0
Jams, jellies (28)	2.8
Milk products (31)	2.0
Nonalcoholic beverages (3)	0.2
Nut products (32)	2.0
Pastas (23)	0.8
Plant protein products (33)	2.0
Processed fruit juices (35)	0.4
Processed vegetable juices (36)	0.4
Snack foods (37)	2.0
Soft candy (38)	1.6
Soup mixes (40)	1.2
Sugar substitutes (42)	4.0
Sweet sauces, toppings, syrups (43)	2.0
White granulated sugar (41)	1.6

[a] Based on 21 CFR §184.1472 (U.S. FDA, 2008) and final rule 70 FR 14530, March 23, 2005.
[1] The number in parenthesis following each food category refers to the paragraph listing of the particular food category in 21 CFR 170.3(n) (U.S. FDA, 2008).

DATA PERTAINING TO SAFETY

Scientific evidence supporting the safety of Ulkenia DHA oil includes a history of ω-3 fatty acid consumption as a result of their natural presence in food, an abundance of published metabolic, clinical, and toxicological data on DHA-containing fish and microalgal-derived oils, and corroborating toxicological and mutagenicity studies on Ulkenia DHA oil, and mutagenicity and toxicity studies on the microalgal source, *Ulkenia sp.*

Studies of Absorption, Distribution, and Metabolism

DHA is an abundant cell membrane fatty acid (18-22), which is obtained directly in the diet or biosynthetically derived through desaturation and elongation of dietary precursor essential fatty acids (20, 23). The absorption, distribution, and metabolism of DHA are known to be similar to other dietary fatty acids. DHA is present in Ulkenia DHA oil in triglyceride form. Following enzymatic hydrolysis of the triglyceride in the upper intestine, the free fatty acids and 2-monoglycerides are incorporated into bile acid micelles for diffusion into the enterocytes, where they are incorporated into new triglycerides (20, 21, 24). Reconstituted triglycerides enter the lymph in the form of chylomicrons and are transported to the blood for distribution and may rapidly be incorporated into plasma lipid fractions, erythrocyte membranes, platelets, and adipose tissue. In passing through the capillaries of adipose tissue and liver, the chylomicron-contained triglycerides and phospholipids are hydrolyzed with subsequent release of free fatty acids to the tissues for metabolism or reesterification into triglycerides and phospholipids for storage as energy (20, 22). Generally, free fatty acids are transported across the mitochondrial membrane in the form of acyl-carnitine (21), where they then undergo β-oxidation with removal of 2 carbons from the fatty acid chain and production of acetic acid, a shorter-chained fatty acid, and acetyl CoA, which combines with oxaloacetic acid and enters the citric acid cycle for energy production (22). As fatty acids of 20 carbons or more are not easily transported across the mitochondrial membrane, DHA also may be metabolized *via* peroxisomal β-oxidation (19, 21, 25-27). As a minor peroxisomal metabolic pathway, DHA may be retroconverted to EPA, which involves β-oxidation, auxiliary enzymes, and removal of 2 carbon units from the carboxyl end of the fatty acid (28-30).

Clinical Studies Relating to the Safety of Ulkenia DHA Oil

Human consumption of dietary DHA from traditional sources is considered safe based on the historical consumption of fish, and fish- and marine-based products. In 1993, the FDA identified 3 possible adverse effects associated with human consumption of ω-3 PUFAs: 1) reduced platelet aggregation; 2) increased low-density lipoprotein (LDL) cholesterol levels; and 3) reduced glycemic control among diabetics [58 FR 2682 (January 6, 1993)] (31).

In 1997, following a thorough evaluation of several published clinical trials, the FDA affirmed that menhaden oil is GRAS under certain specified conditions of intended use (21 CFR §184.1472), and concluded that consumption of up to 3 g DHA + EPA combined/person/day does not pose a "significant risk for increased bleeding time", has no "clinically significant effect on glycemic control", and is "safe with respect to the effect on LDL cholesterol" (15). Subsequently, in a public letter permitting a dietary supplement qualified health claim for ω-3 fatty acids and coronary heart disease, FDA stated that the use of EPA and DHA ω-3 fatty acids, including DHA from marine microalgae, as dietary supplements is safe (13).

Subsequent to FDA's 1997 GRAS affirmation of menhaden oil, a number of additional clinical studies of the potential effects of DHA-containing oils on hemostatic parameters, glycemic control, and LDL-C have been conducted. A comprehensive summary of greater than 90 published clinical trials was reviewed by the Panel (32-135). These clinical studies ranged in duration from 1 week to >1 year, and were conducted with DHA-containing fish and marine oils providing up to 6 g DHA/day, alone or in combination with DPA and/or EPA. The available clinical data do not provide evidence that DHA, at estimated exposures provided through the intended use of Ulkenia DHA oil, would have an adverse effect on human health. The clinical data measured endpoints such as LDL-C levels, glycemic control, bleeding time, platelet aggregation, or other hemostatic parameters, as well as immune function or response, kidney or liver function, or lipid peroxidation. Overall, the results of these studies indicate that DHA, provided in fish or marine-derived oils alone or in combination with DPA and/or EPA, at levels up to 6 g DHA/person/day, would not produce significant adverse effects on these parameters. These results are consistent with the earlier conclusions of the FDA that consumption of up to 3 g DHA + EPA/day is safe.

The Panel noted that consumption of DHA-containing oils providing up to 6 g DHA/day, alone or in combination with DPA and/or EPA, has been reported to result in alterations of platelet and total serum phospholipid and nonesterified fatty acid compositions, with increases in the levels of ω-3 PUFAs, including DHA, and concomitant decreases in levels of arachidonic acid (AA) (20:4; ω-6) of up to 26% (42-45, 48, 56, 58-60, 72, 73, 77, 81, 82, 84, 113, 124, 136). Arachidonic acid (AA), either obtained in the diet or as a biosynthetic product of linoleic acid (18:2; ω-6), occurs as a fatty acid component of platelet membranes and most tissue phospholipids functioning as the main precursor of eicosanoids, which are involved in mediation of hemostatic parameters and immune cell function and response (20, 137, 138). Critical review of the scientific literature indicates that while AA levels may decrease following consumption of ω-3 PUFAs, the concentration of AA generally remains within normal physiological concentrations of 5 to 15% of total fatty acids of platelet membranes and tissue phospholipids. Additionally, the fatty acid composition of Ulkenia DHA oil comprises up to 1.8% AA. There is no indication that DHA, at exposures estimated through the proposed uses of Ulkenia DHA oil, would adversely affect hemostatic parameters or immune function or response as a result of

possible concomitant decreases in platelet and total serum phospholipid and nonesterified fatty acid levels of AA (42-44, 46, 48, 56, 58-60, 72, 73, 77, 81, 82, 84, 124-128, 136).

When viewed in its entirety, the scientific evidence from published clinical studies with DHA-containing oils indicates that the consumption of Ulkenia DHA oil, under the conditions of intended use, would not be expected to produce adverse effects on human health.

Toxicological Studies

Safety of Other DHA-Containing Oils

The safety of DHA-containing fish and microalgal-derived oils has been studied extensively. Overall, the data from these toxicological studies indicate that gavage or dietary administration of fish or microalgal-derived oils does not produce significant adverse effects on mortality, body weight gains, food consumption, or clinical observations in laboratory animals including mice, rats, and pigs (25, 139-150). Yellow fat disease, which is characterized by accretion of lipofuscin pigment, degeneration of adipose cells (steatosis), and inflammation of adipose tissue (steatitis) (139, 151, 152), has been reported to occur naturally in wildlife species and was reported in rats, rabbits, mink, and pigs in various toxicological studies following the consumption of diets rich in ω-3 PUFAs (*i.e.*, ω-3 PUFA-containing oils at dietary concentrations ranging from as high as 12 to 19% for periods of 8 weeks to 12 months in length) in combination with a vitamin E deficient state (139, 140, 142, 147, 151-159). This effect is considered a normal response to a large load of dietary lipids, and may be prevented by concurrently administering vitamin E with high-PUFA diets (154, 158-161). Some study authors reported increased liver and spleen weights in mice and rats administered high PUFA diets at daily intakes ranging from 25 to 9,500 mg/kg body weight and varying durations of 4 to 13 weeks (139, 141, 142, 144, 147, 148, 162, 163); however, no histopathological effects were observed in these organs and the increased organ weights were reported to be adaptations to accommodate the large lipid load.

Studies of Ulkenia DHA Oil

In addition to a critical review of the available scientific data on the safety of DHA-containing fish and other microalgal-derived oils, the safety of Ulkenia DHA oil (reported as DHA45-oil) derived from *Ulkenia sp.* was assessed in various genotoxicity and acute, subchronic, and reproductive toxicity studies. These studies were published by Blum *et al.* (204, 205) and summarized and reported by Kroes *et al.* (201).

Ulkenia DHA oil (reported as DHA45-oil) was not genotoxic in the Ames assay using *Salmonella typhimurium* and *Escherichia coli* WP2 *uvrA*, with or without metabolic activation (164, 165, 201, 204), or in a chromosomal aberration assay in Chinese hamster fibroblast cells (166, 201, 204). Ulkenia DHA oil (reported as DHA45-oil) was reported to have low acute oral toxicity in mice and rats (*i.e.*, LD_{50} values >2,000 mg Ulkenia DHA oil/kg body weight) (167, 168, 201, 204).

In a subchronic toxicity study of Ulkenia DHA oil (reported as DHA45-oil), groups of 30 Sprague-Dawley Crj:CD (SD) IGS rats (15/sex/group) were administered distilled water or various combinations of Ulkenia DHA oil and DHA27 (an oil containing 27% DHA) by daily oral gavage for a period of 90 days (169, 201). The combinations of Ulkenia DHA oil/DHA27 tested included 0/2,000, 500/1,500, 1,000/1,000, and 2,000/0 mg/kg body weight/day. Based on total DHA oil content, these dosing regiments provided daily doses of 540, 630, 720, and 900 mg DHA/kg body weight/day, respectively. Additional groups of 5 rats/sex/dose receiving distilled water or 2,000 mg/kg body weight/day of Ulkenia DHA oil or DHA27 were allowed a 4-week recovery period following the 90 days of dosing. Effects of treatment on mortality and clinical signs, neurologic responses, body weight gain, food and water consumption, hematology, clinical chemistry, urinalysis, and on the results of ophthalmology, gross pathological, and histopathological examinations were evaluated. There were no treatment-related differences between DHA27 and Ulkenia DHA oil. Compared to the water controls, there were no biologically significant effects of treatment with Ulkenia DHA oil alone or in combination with DHA27. At necropsy, increased relative liver weights were reported in animals fed Ulkenia DHA oil alone or 1,000 mg/kg body weight/day of each oil, which were likely the result of the large lipid load, as reported for rats in several published feeding and gavage studies with fish and marine algal-derived oils providing daily intakes ranging from 25 to 9,500 mg/kg body weight for durations of 4 to 13 weeks. In the absence of histopathological lesions or changes in enzymes indicative of liver toxicity, the increased liver weights were considered to be not toxic in nature. Similarly, reported increases in the absolute weights, and in some cases, relative weights, of several organs including the spleen, kidneys, and adrenals were not considered to be of toxicological significance as there were no histopathological correlates to the organ weight findings and, in all cases, the magnitude of change was small. Thus, 90-day gavage treatment with Ulkenia DHA oil, alone or in combination with DHA27 was reported to have no adverse effect on the findings of the ophthalmology, gross pathology, or histopathology examinations. Additionally, dose-dependent effects were not observed with increasing levels of DHA administration. Given the lack of histopathological effects, the NOAEL in this study was considered to be 2,000 mg Ulkenia DHA oil/kg body weight/day (approximately 900 mg DHA/kg body weight/day), which was the highest dose tested.

The *in utero* toxicity of Ulkenia DHA oil (reported as DHA45-oil) produced from *Ulkenia sp.* was evaluated in a one-generation study in rats (170, 201, 205). Groups of 56 Wistar (Crl:(WI)WU BR) rats (28/sex/group) were administered Ulkenia DHA oil in the diet at concentrations of 0 (control), 1.5, 3.0, or 7.5% for 10 weeks prior to mating. The total intake of test material (as Ulkenia DHA oil) ranged throughout the different phases of the study period and is presented in Table 3. After successful mating, males were sacrificed and necropsied, while females continued to be exposed at their respective concentrations and were allowed to deliver. On post-natal Day (PND) 21, all pups were weaned and sacrificed after gross external evaluation. Evaluations were made of mortality, clinical signs, body weights, food consumption, fertility and reproductive performance, litter size, malformed pups, pup weights, and gross pathology and

histology of dead or stillborn pups and all F_0 animals. There was no effect of treatment with Ulkenia DHA oil on mortality or clinical signs. Sporadic increases in body weight gains were reported for some of the treatment groups compared to corn oil controls; however, differences in food consumption also were detailed, which the authors indicated may have accounted for the observations of weight gains in these groups. With respect to reproductive parameters, treatment with Ulkenia DHA oil was reported to have no effect on precoital time, mating index, fertility indices, fecundity, gestation index and duration, numbers of stillborn pups, or post-implantation losses. Similarly, there were no adverse effects of treatment on the numbers of pups, pups/litter, pup mortality, sex ratio, pup weight, or incidence of malformations. Absolute liver weights were increased in mid- and high-dose males, and in low- and high-dose females; however, these effects were considered by the study authors to be of no toxicological significance, as no gross or histopathological correlates were reported and similar effects have been reported in rats in several feeding and gavage studies of fish and marine algal-derived oils. Increased absolute and relative spleen weights were reported and were associated with an increased incidence and/or severity of extramedullary hematopoiesis observed histologically. The only significant gross pathology reported was the presence of "yellow spots" within the abdominal adipose tissue of the F_0 animals. These yellow spots were reportedly compatible with findings associated with yellow fat disease, and microscopic evaluation of these yellow spots yielded the diagnosis of lipogranuloma by the study authors. To address these reported effects, a panel of internationally recognized expert pathologists (Expert Pathology Panel) was convened to independently and collectively critically evaluate the scientific data of this study, as well as additional relevant data compiled from the published literature and any other data and information deemed by the pathology experts to be pertinent to the evaluation. The Expert Pathology Panel agreed that the reported effects were related to the administration of high levels of Ulkenia DHA oil (reported as DHA45-oil), but, they concluded that the adipose tissue alteration was in fact steatitis, which occurs naturally and experimentally in many different species fed diets that are high in PUFAs, including DHA-containing oils, and are relatively low in antioxidants (171). The Expert Pathology Panel unanimously concluded that the steatitis was the "result of a nutritional imbalance… attributable to the extremely high levels of DHA45-oil administered", the increased spleen weights were due to extramedullary hematopoiesis "consistent with a response to steatitis," and the "findings reported in the one-generation reproduction study are those expected with high exposure to PUFAs and raise no concern for human safety of DHA45-oil under appropriate conditions of use." The findings of the Expert Panel were reported by Kroes *et al.* (201).

Safety of Other Components of Ulkenia DHA Oil

Palmitic Acid

The total fatty acid content of Ulkenia DHA oil comprises approximately 33% palmitic acid (16:0) of total fatty acids. Palmitic acid is a natural component of the human diet and has been identified in meat, poultry, fish, grain products, and milk and milk products (172). Palmitic acid was reported to have an estimated daily intake in the U.S. population in the range of 11 to 19 g/day (172).

The exposure to DHA from the intended food uses will be a maximum of 3 g/day under the conditions of intended use. On the basis of Ulkenia DHA oil containing a minimum of 43% DHA of the total fatty acid content, the maximum intake of palmitic acid corresponds to approximately 2.3 g/day. This intake is at least 5 times less than the amount of palmitic acid already consumed as a natural constituent of the diet, and thus the estimated intake of palmitic acid through the intended use of Ulkenia DHA oil would not have an impact on the relative amount of daily dietary palmitic acid. Therefore, the estimated intake of palmitic acid through the proposed uses of Ulkenia DHA oil would not be expected to produce adverse effects on human health.

DPA

The fatty acid composition of Ulkenia DHA oil includes up to 14 and 0.6%, respectively, of the ω-6 and ω-3 isomers of DPA. Similar to DHA, both forms of DPA are cell membrane fatty acids (23, 29, 173-175) which may be derived by biosynthesis through elongation, desaturation, or shortening of endogenous long chain fatty acids (20, 23, 27-29), or by the dietary intake of foods such as fish, seal, or fish or microalgal-derived oils (44, 176, 177). As both DPA isomers are component fatty acids of fish oil, the safety of ω-3 and ω-6 DPA is supported by the numerous non-clinical and clinical studies that have reported no adverse effects following dietary supplementation with fish or marine oil.

In a series of dietary studies in rats and rabbits that evaluated the subchronic, reproductive, and developmental toxicity of microalgal-derived DPA (ω-6)-containing oils (calculated to provide doses of up to 630 mg DPA/kg body weight/day) (149, 150, 178), no compound-related effects on survival, clinical observations, body weight gains, food consumption, urinalysis measurements, or gross necropsy findings, and no effect of treatment on hematological parameters, spleen weight, or on the gross and histopathological appearance of the spleen or adipose tissue were reported. Overall, there were no reported effects of treatment on reproductive performance, duration of gestation, mean litter size, or number of litters with live and/or dead pups, and no effects on postimplantation loss, mean fetal or pup body weight/litter, or morphological developments in either species.

In zinc deficient and non-deficient rats provided a dietary dose of up to 17 mg DPA/kg body weight for a period of 6 weeks, no differences between non-zinc deficient control or treatment groups with respect to final body weight, absolute liver and testes weight, plasma and testes zinc levels, or spermatid content of the testis parenchyma were reported (173).

Additionally, as described previously, subchronic toxicity and one-generation reproduction studies were performed in rats using Ulkenia DHA oil (reported as DHA45-oil) (201, 204, 205). In the 90-day subchronic toxicity study, gavage administration of combinations of Ulkenia DHA oil/DHA27 to groups of 30 Sprague-Dawley Crj:CD (SD) IGS rats (15/sex/group) provided daily combined ω-3 and ω-6 DPA doses of at least 0, 41, 82, and 163 mg DPA/kg body weight/day (169, 201, 204). As described previously, there were no biologically significant effects of treatment with Ulkenia DHA oil at the highest dose evaluated in the study, 2,000 mg Ulkenia DHA oil/kg body weight/day, providing 163 mg DPA/kg body weight/day. In the one-generation reproductive toxicity study in Wistar (Crl:(WI)WU BR) rats (28/sex/group), DPA intakes ranged from 65 to 913 mg DPA/kg body weight/day (see Table 3), without adverse toxicological effects attributable to Ulkenia DHA oil, or components thereof (170, 201,205). As discussed above, lipogranuloma of the abdominal adipose tissue and increased absolute and relative spleen weights associated with an increased incidence and/or severity of extramedullary hematopoiesis were reported in some of the treatment animals compared to controls, and it was unanimously concluded by the Expert Pathology Panel that the "findings reported in the one-generation reproduction study are those expected with high exposure to PUFAs and raise no concern for human safety of DHA45-oil under appropriate conditions of use" (201, 205).

Table 3 Estimated Daily Intake of Ulkenia DHA oil, DHA, and DPA[1] by Rats in the One-Generation Reproduction Study

	Low-Dose Group (1.5% Ulkenia DHA oil)	Mid-Dose Group (3.0% Ulkenia DHA oil)	High-Dose Group (7.5% Ulkenia DHA oil)
	Estimated Intake of Ulkenia DHA oil (mg/kg body weight/day)		
Males	800 to 1,000	1,500 to 2,000	3,400 to 4,700
Females (pre-mating)	1,800 to 2,200	3,400 to 4,300	7,900 to 9,700
Females (gestation and lactation Days 1 to 14)	1,800 to 2,700	3,700 to 5,300	7,800 to 11,200
	Estimated Intake of DHA (mg/kg body weight/day)		
Males	360 to 450	675 to 900	1,530 to 2,115
Females (pre-mating)	810 to 990	1,530 to 1,935	3,555 to 4,365
Females (gestation and lactation Days 1 to 14)	810 to 1,215	1,665 to 2,385	3,510 to 5,040
	Estimated Intake of DPA (mg/kg body weight/day)		
Males	65 to 82	122 to 163	277 to 383
Females (pre-mating)	147 to 179	277 to 350	644 to 791
Females (gestation and lactation Days 1 to 14)	147 to 220	302 to 432	636 to 913

[1] Estimated DPA values were calculated using a minimum value of 8.15% as the DPA (ω-6 + ω-3) content of Ulkenia DHA oil.

Although no clinical studies were identified that evaluated the possible effects of DPA alone, 6 studies of fish or marine-derived oils with reported DPA contents were reviewed and provide supporting evidence for the safety of dietary DPA. No significant effects on glycemic control or LDL-C levels were reported in healthy subjects or diabetic patients receiving supplemental fish oil providing up to 800 mg DPA/day for periods of 6 weeks to 9 months (approximately 13 mg DPA/kg body weight/day for an average 60 kg individual) (54, 56, 84, 98, 99). Decreased collagen-induced platelet aggregation was reported in 18 diabetic patients receiving fish oil providing 200 mg DPA/day for 6 weeks; however, ADP-induced aggregation was not affected and therefore the Panel considered that the overall effect of the fish oil on platelet aggregation is not clear (54). Increased bleeding times and decreased von Willebrand factor were reported in 24 healthy subjects (10 males, 14 females) consuming fish oil providing approximately 210 mg DPA/person/day for a period of 9 months (84). Although bleeding times were increased, fibrinogen levels also were increased and there was no associated reduction in platelet aggregability; thus, the clinical significance of the increased bleeding times is not clear. Additionally, due to the various ω-3 and ω-6 fatty acids comprising the fatty acid content of fish oils, the effects reported by Schmidt *et al.* (84) and Axelrod *et al.* (54) cannot be attributed to a single PUFA, such as DPA. In 12 hyperlipidemic patients (10 males, 2 females) receiving 0.42

or 0.84 mL DPA/day (approximately 0.39 and 0.78 mg, respectively)[1] for a period of 4 weeks, LDL-C levels were decreased by 2% and no significant effects on glycemic control were reported (44). Considering the totality of available evidence, the estimated intake of DPA (<1 g/day) through the proposed uses of Ulkenia DHA oil would not be expected to produce adverse effects in humans.

Sterols

The unsaponifiable fraction of Ulkenia DHA oil is low and consists primarily of sterols at a level around or even below 1%. Representative lot analyses of Ulkenia DHA oil identified the main sterol components as cholesterol, 7-dehydrostigmasterol and 4-methyl-chondrillasterol.

The safety of dietary cholesterol and other sterols is well documented in the scientific literature. Cholesterol is a natural constituent of the human diet, and 4-methyl sterols have been identified in the normal metabolic pathway of cholesterol biosynthesis in man, and in several food sources, including fish, shellfish, and rice bran (180, 181). 7-Dehydrostigmasterol was quantified in phytosterols purified from the unsaponifiable fraction of soybean oil (182). Hence, although quantitatively minor constituents, the sterols in Ulkenia DHA oil would be considered to already occur in the human diet.

With typical sterol levels below 1% in Ulkenia DHA oil, the level of intake of sterols under the intended conditions of use of Ulkenia DHA oil would be minimal. Typical cholesterol levels are around 30% of total sterols. Assuming a maximum intake of Ulkenia DHA oil of 7.9 g/day (based on 38% DHA in Ulkenia DHA oil contributing to 3 g/person/day), a total sterol content of 1% and 30% of sterols being cholesterol, the theoretical maximum daily cholesterol intake from Ulkenia DHA oil would be approximately 24 mg. Similarly, the theoretical maximum intake for phytosterols would be approximately 55 mg/day. The safety of dietary cholesterol and phytosterols is well documented in the scientific literature. Cholesterol and phytosterols are natural constituents of the diet and 4-methyl sterols occur in the endogenous metabolic pathway of cholesterol biosynthesis. These intakes are well below the amounts of sterols already consumed as natural constituents of the diet (up to 400 mg/person/day) (183-187), and thus, the estimated intake of sterols through the proposed uses of Ulkenia DHA oil would not have an impact on the relative amount of cholesterol and phytosterols already consumed in the diet. Therefore, the dietary exposure to cholesterol, 7-dehydrostigmasterol, and 4-methyl-chondrillasterol from the intended use of Ulkenia DHA oil would not be expected to produce adverse effects on human health.

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[1]Calculated based on the density value for menhaden fish oil (179)

Studies Pertaining to the Safety of the Source Organism

The source organism (a thraustochytrid) is a member of the kingdom Chromista (188-191). Although there are no data demonstrating detailed human consumption of the source organism, thraustochytrids have been identified in plankton and other marine detritus and comprise a portion of the diet of filter-feeding invertebrates, and thus constitute an indirect component of the human diet through consumption of fish and other marine animals (192-195). There have been no identified reports of *Ulkenia sp.* producing toxic chemicals or pathogenicity (196, 197). Microalgal toxin analysis of representative lots of Ulkenia DHA oil confirmed the absence of the common algal and cyanobacterial toxins, such as domoic acid, Paralytic Shellfish Poisons (PSP), Diarrhetic Shellfish Poisons (DSP), Neutrotoxic Shellfish Poisons (NSP), pectenotoxins, yessotoxins, azaspiracides, Prymnesium toxins, and microcystins and nodularin. Additionally, independent scientific expert evaluation of the available data pertinent to the safety of the microalgal source concluded *Ulkenia sp.* is both nontoxigenic and nonpathogenic to man (198, 203).

The conclusion that the microalgae is nontoxicogenic is supported by mutagenicity and classical rodent toxicity studies using the Ulkenia DHA oil source organism. The microalgal source was reported to produce negative results in the Ames assay using *Salmonella typhimurium* strains TA97, TA98, TA100, and TA102, with or without metabolic activation (199). In a 14-day feeding study, the microalgal source suspended in distilled water was administered orally to ICR male mice (SPR) at a dose of 2 g source organism/kg body weight/day (200). A control group received distilled water only. No significant differences in clinical signs, body weight gains, or necropsy observations were reported for the microalgal source-treated mice.

Summary

Overall, when viewed in its entirety, the scientific data summarized above support the safe intake of Ulkenia DHA oil under the conditions of intended use in traditional foods. While the production of Ulkenia DHA oil from the microalgae *Ulkenia sp.* for use as a food ingredient is unique, the source microorganism is nontoxigenic and nonpathogenic, and all components of Ulkenia DHA oil have a history of occurrence in the diet. The safety of Ulkenia DHA oil is established by a wealth of historical information on populations consuming higher levels of DHA than those that would result from the conservative estimate of intake under the conditions of intended use. Clinical studies reveal no potential for toxicity of DHA under the conditions of intended use. The epidemiologic and clinical data are supported by animal toxicity studies, which are corroborated by studies on Ulkenia DHA oil (reported as DHA45-oil). The Panel noted the growing body of evidence of cardiovascular risk reduction with the use of ω-3 fatty acids in randomized placebo-controlled clinical trials.

There are no indications from the published literature, or from the published studies conducted with Ulkenia DHA oil, that under the conditions of intended use in traditional foods, Ulkenia DHA oil would result in any adverse health effects.

CONCLUSION

Based on our independent and collective, critical review of the available pertinent scientific evidence, as members of an Expert Panel specially convened for this purpose, we unanimously conclude that DHA-rich oil, meeting appropriate food grade specifications, and manufactured and used in accordance with current good manufacturing practice, under the conditions of intended use specified herein, is "generally recognized as safe" (GRAS), based on scientific procedures.

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CONCLUSION

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TAB 2

TAXONOMY AND SAFETY OF *ULKENIA* (TAB 2)

Ulkenia SAM 2179 strain is a wild type strain. It was deposited with the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology (address: 1-3, Higashi 1 chome Tsukuba-shi Ibaraki-ken 305, JAPAN), on July 23, 1996.

Ulkenia is a genus of marine protist (or microalgae), which was first discovered by A. Gaertner and S. Raghu Kumar in 1977 (European Register of Marine Species, 2001). *Ulkenia* sp. belongs to the Thraustochytriaceae family, also referred to as thraustochytrids, which includes the following 6 genera: *Aplanochytrium, Japonochytrium, Althornia, Thraustochytrium, Schizochytrium*, and *Ulkenia* (Bahnweg, 1979a; Honda *et al.*, 1999; European Register of Marine Species, 2001). Initially, a separate genus, Labyrinthuloides, was identified as belonging to the Thraustochytriaceae family; however, Leander and Porter (2000), determined that this genus was synonymous with Aplanochytrium, and therefore transferred 5 species of Labyrinthuloides and 1 species of Labyrinthula to the genus Aplanochytrium. *Ulkenia* genus may be further subdivided into the following species: *U. profunda, U. radiata, U. sarkariana, U. minuta, and U. visurgensis* (Honda *et al.*, 1999; European Register of Marine Species, 2001).

The taxonomy of the current Thraustochytriaceae family has undergone various modifications since its original classification as a fungus (Cavalier-Smith *et al.*, 1994). Thraustochytrids were originally recognized as a fungus of the order Chytridiales, but due to their structure, were reassigned to the Oomycete order, Saprolegniales (Cavalier-Smith *et al.*, 1994; Honda *et al.*, 1999). The addition of new genera prompted segregation of the thraustochytrids into their own order, Thraustochytriales, and based on their similarity to the labyrinthulids and recent discoveries made through 18S-rRNA-gene sequencing, the thraustochytrids were subsequently removed from the Oomycetes class and classified with the labyrinthulids in the class Labyrinthulea or Labyrinthulomycetes (Cavalier-Smith *et al.*, 1994; Honda *et al.*, 1999). Labyrinthulea were further grouped under Heterokonta within the Chromista kingdom. Thraustochytrids have also been further classified under a separate protist phylum, Labyrinthulomycota; however, it is not clear whether or not this name has been validated under the Botanical Code (Cavalier-Smith *et al.*, 1994; Honda *et al.*, 1999). A summary of the current taxonomic assignment (based on the 8 kingdom classification scheme) of *Ulkenia* sp. is presented in Table A-1.

Table A-1 Summary of the Taxonomic Assignment of *Ulkenia* sp.	
Taxonomy	**Taxonomic Assignment**
Kingdom:	Chromista
Subkingdom	Heterokonta
Phylum:	Labyrinthulomycota
Class:	Labyrinthulea (Labyrinthulomycetes)
Order:	Thraustochytriales
Family:	Thraustochytriaceae
Genus:	*Ulkenia*
Species	*Ulkenia* sp. SAM 2179

Cavalier-Smith *et al.*, 1994; Honda *et al.*, 1999

The Chromista kingdom, also referred to as Stramenophiles, incorporates a divergent evolutionary line from the same ancestor as plants, fungi, and animals (Waggoner and Speer, 2001). Chromista are tubulocristate protists defined by a distinct subset of tripartite tubular hairs, which usually occur on the flagella, and include diatoms, water molds, kelp, coccolithophorids, bicoeceans, slime nets, silicoflagellates, and golden, brown, and yellow-green algae (Sogin and Patterson, 1995; Waggoner and Speer, 2001).

Thraustochytrids are present in marine and estuarine environments and have been isolated from coastal plankton and marine macrophytic detritus (Ulken *et al.*, 1990; Sathe-Pathak *et al.*, 1993; Naganuma *et al.*, 1998). *Ulkenia* sp. has been identified and enumerated in decomposing algal tissues of the brown alga Sargassum cinereum J. Ag., and due to their saprotrophic manner, it has been proposed that thraustochytrids play a functional role in marine detrial dynamics, utilizing ectoplasmic net elements to penetrate a wide range of organic particles for vital nutrients, such as carbohydrates, phenols, proteins, reducing sugars, and cellulose (Bahnweg, 1979a,b; Sathe-Pathak *et al.*, 1993; Raghukumar *et al.*, 1995). Additionally, *Ulkenia* sp. might play an important role in marine ecology as they were suggested to induce the settlement of barnacle larvae to surfaces of glass, aluminum, mild steel, and fiberglass panels immersed in seawater for periods of 1 to 4 days (Raghukumar *et al.*, 2000; Huang *et al.*, 2001).

To date, there are no data demonstrating detailed human consumption of thraustochytrids, or more specifically, *Ulkenia* sp. However, thraustochytrids exhibit worldwide distribution and have been identified in areas such as the west Pacific Ocean, the White Sea, and the Mediterranean (Ulken *et al.*, 1990; Sathe-Pathak *et al.*, 1993; Bongiorni, 1998; Honda *et al.*, 1998; Naganuma *et al.*, 1998). Thraustochytrids have been identified in plankton and other marine detritus and comprise a portion of the diet of filter-feeding invertebrates in the marine ecosystem (Sathe-Pathak *et al.*, 1993; Azevedo and Corral, 1997; Naganuma *et al.*, 1998). Thraustochytrids would therefore constitute an indirect component of the human diet through consumption of fish and other marine animals, such as clams or mussels.

The safety of *Ulkenia* sp. used in the production of Ulkenia DHA oil is based on the expert opinions of Dr. Karsten Schaumann and Prof Dr. M. Melkonian (see following). The expert opinion of Schaumann (2003), "Extended Expert's Report on the Safety of *Ulkenia* sp.", was based on experience in scientific dealings with *Ulkenia*, findings on taxonomy and phylogeny, as well as biology, ecology, physiology, and toxicology of the genus *Ulkenia* and related taxa in the published literature. Schaumann (2003) concluded that algal toxins are not expected to occur in docosahexaenoic acid (DHA) obtained from *Ulkenia* sp. and that products extracted from *Ulkenia* sp., such as DHA oil, pose no dangers to human health. Similarly, Melkonian (2003) based his expert report, "Expert's Report on the Safety of *Ulkenia* (Thraustochytriales) in a Human-biological Context", on his own knowledge, as well as an extensive literature review of the occurrence of toxins in the Thraustochytriales and in *Ulkenia*. Prof Dr. M. Melkonian concluded that the oil extracted from *Ulkenia* does not contain algae toxins based on the extensive literature demonstrating the absence of the production of toxins in *Ulkenia*, the order Thraustochytriales gave no indication as to the presence of any toxic components (Melkonian, 2003). Furthermore, toxins known to be present in algae/Stramenopila occur only in photosynthetic or mixotrophic organisms, which are not phylogenetically related to *Ulkenia*, or its phylum Labyrinthulea. Both Schaumann (2003) and Melkonian (2003) concluded that *Ulkenia* sp. used in the production of Ulkenia DHA oil is non-pathogenic and non-toxicogenic.

To further characterize the safety of *Ulkenia* sp. used in the production of Ulkenia DHA oil, 3 lots of Ulkenia DHA oil were analyzed for the presence of algal and cyanobacterial toxins that that have been identified in the published literature and mentioned in international food regulations using liquid chromatography-mass spectrometry (LC-MS). Domoic acid, Paralytic Shellfish Poisons (PSP), Diarrhetic Shellfish Poisons (DSP), and microcystins/nodularin were not detected at detection limits of 100, 50, 10, and 100 µg/kg, respectively.

Additional analyses have been conducted with 5 lots of *Ulkenia* sp. biomass and 2 lots of Ulkenia DHA oil for domoic acid (detection limit 100 µg/kg), DSP toxins (detection limit 50 µg/kg), pectenotoxins (detection limit 50 µg/kg), yessotoxins (detection limit 50 mg/kg), azaspiracides (detection limit 50 µg/kg), PSP toxins (detection limit 10 µg/kg), Neurotoxic Shellfish Poisons (NSP) toxins (detection limit 50 µg/kg), microcystins and nodularin (detection limit 100 µg/kg). None of these toxins were detected in any of the samples, biomass or oil. An *Artemisia* assay for Prymnesium toxins indicated no evidence of these toxins in the *Ulkenia* sp. biomass or Ulkenia DHA oil.

TAB 3

FATTY ACID COMPOSITION OF ULKENIA DHA OIL (TAB 3)

Lipid-analysis of Ulkenia DHA oil is conducted in accordance with validated and approved official methods, as recommended by the German Government, the American Oil Chemists Society (AOCS) or the Deutsche Gesellschaft für Fettwissenschaft (DGF) (German Society for Fat Science). Similarly, the fatty acid composition of Ulkenia DHA oil (Table A-2) is determined through validated and approved official methods, as recommended by the German Government, the AOCS (AOCS Official Method Ce 1e-91; 1993), or the DGF.

Table A2 The Fatty Acid Composition of Ulkenia DHA Oil Intended for Use as a Food Ingredient

Fatty acid content (% of total fatty acids)	Lot 990222	Lot 110708	Lot 120306
Tetradecanoic acid (Myristic acid) (14:0)	2.4	2.9	2.7
Pentadecanoic acid (15:0)	1.4	1.6	1.2
Hexadecanoic acid (Palmitic acid) (16:0)	32.2	33.5	33.1
Heptadecanoic acid (17:0)	0.5	0.5	0.4
Octadecanoic acid (18:0)	1.1	1.0	1.1
Eicosatetraenoic acid (20:4, ω-6)	1.1	1.2	0.9
Eicosatetraenoic acid (20:4, ω-3)	0.8	0.7	0.8
Docosapentaenoic acid (22:5, ω-6)	11.6	10.5	11.4
Docosapentaenoic acid (22:5, ω-3)	0.3	0.3	0.3
Docosahexaenoic acid (22:6, ω-3)	46.2	45.3	45.3
Others[a]	2.7	2.7	3.0
Sum	100.0	100.0	100.0

[a] Other fatty acids identified by GC-MS as minor components of Ulkenia DHA oil include: 10:0, 10:1, 11:0, 12:0, 13:0, 14:1, 14:2, 16:1, 16:2, 16:3, i-17:0, 18:2, 18:3, 18:4, 19:0, 20:0, 20:3, 20:5, 22:0, 24:0, and 26:0. No erucic acid (22:1, n-9) is present and trans-fatty acids were not detected

The analysis of triacylglycerol species of Ulkenia DHA oil is conducted by high-performance liquid chromatography (HPLC) (AOCS Official Method Ce 5b-89; 1993). To determine the distribution of fatty acids of Ulkenia DHA oil (Lot. 120306) in the glycerol backbone, ^{13}C-NMR spectrometry analyses were performed according to Haraldsson *et al.* (2000) (Table A-3).

Table A-3 Analyses of Isolated Triacylglycerols of Ulkenia DHA Oil by ^{13}C-NMR Spectrometry

HPLC Peak No.	Triacylglycerol Species	NMR Sample Identifier	Triacylglycerol Species Confirmed
2	DHA:DPA:DHA	FY124-2	Yes
4	DHA:DHA:C16	FY124-3	Yes
5	DHA:DPA:C16 DHA:DHA:C16	FY124-4	No
6	C16:DHA:C16	FY124-5	Yes
7	C16:DHA:C16 C16:DPA:C16	FY124-6	Yes
Standard[a]	DHA:DHA:DHA	FY124-1	-
Standard[a]	C16:C16:C16	FY124-7	-

[a] Available from Sigma-Aldrich®

The quantification of triacylglycerol species of Ulkenia DHA oil also is conducted by HPLC (AOCS Official Method Ce 5b-89; 1993). The main triacylglycerols of Ulkenia DHA oil, by percent composition, are provided in Table A-4.

Table A-4 The Main Triacylglycerol Species of Ulkenia DHA Oil

Triacylglycerol species (% of total triacylglycerols)	Lot 990222	Lot 110708	Lot 120306	Range
DHA:DHA:C16[a]	26.1	24.5	26.3	20.0-30.0
C16:DHA:C16	20.0	17.4	16.5	14.0-23.0
DHA:DPA:C16	14.0	12.0	12.7	8.0-16.0
DHA:DHA:DHA	6.8	6.8	7.4	5.0-10.0
DHA:DPA:DHA	4.4	5.0	4.8	3.0-8.0
C16:DPA:C16	5.2	4.3	5.2	1.5-8.0
DHA:C14:DHA[a]	3.0	3.5	3.3	1.0-5.0
C16:C16:C16	1.7	2.8	2.9	0.8-4.0
Others	18.8	23.7	20.9	15-30

[a] Positions of the fatty acids not determined

The unsaponifiable fraction of Ulkenia DHA oil is generally below 1.4% and is made up primarily of sterols, which are, however, present at levels below 1%. These sterols have been identified as sterines, 4-methylsterines, and triterpenes by GC-MS and Nuclear Magnetic Resonance (NMR)-spectroscopy. The total sterol content of 3 non-consecutive representative lots of Ulkenia DHA oil intended for use as a food ingredient are provided in Table A-5.

Table A-5 Sterol Content of Ulkenia DHA Oil

	Method	Lot 990222	Lot 110708	Lot 120306
Sterol content (%)	ISO/FDIS 12228:1999(E)	0.179	0.216	0.306

In addition to cholesterol, several 4-methyl sterols were identified in Ulkenia DHA oil. The main sterols of Ulkenia DHA oil, isolated by silver column chromatography and identified by GC-MS and NMR spectroscopy are presented in Table A-6. The three main sterols were identified as cholesterol, 24-ethyl-cholesta-5,7,22-trien-3-ol (7-dehydrostigmasterol) and 4-methyl-24-ethyl-cholesta-7,22-dien-3-ol (4-methyl-chondrillasterol).

Table A-6 The Main Sterols of Ulkenia DHA Oil

Sterol	Molecular weight (g/mol)	Molecular weight acetates (g/mol)	Molecular weight TMS (g/mol)	Content % (of total sterol, TMS-derivatives)		
				Lot 990222	Lot 110708	Lot 120306
Cholest-5-en-3-ol (Cholesterol)	386	428	458	19.4	19.1	30.7
24-Ethyl-cholesta-5,7,22-trien-3-ol (7-Dehydrostigmasterol)	410	452	482	33.5	28.4	29.3
4-Methyl-24-ethyl-cholesta-7,22-dien-3-ol (4-Methyl-chondrillasterol)	426	468	498	14.5	18.5	14.2
Others				32.6	34.0	25.8

The formation of trans-fatty acids is known to result from exposure of unsaturated fatty acids to high temperatures (*e.g.*, in the deodorization step). As the presence of trans-fatty acids is undesirable from a nutritional point of view, refining conditions, especially conditions of deodorization were optimized in the course of product development of Ulkenia DHA oil. Table A-7 shows results of analyses for trans-fatty acids and demonstrates that the trans fatty acid content of Ulkenia DHA oil is in compliance with the specifications outline in Table 3 part II of the notification. The values are reported on the basis of total fatty acids, while the limit in the specifications applies for the oil itself. These values for trans-fatty acids were determined by indirect calculation using GC of fatty acids following the AOCS Official Method Cd 14c-94.

Table A-7 Trans Fatty Acids in Ulkenia DHA Oil

Value	Method	Lot 990222	Lot 110708	Lot 120306
Trans Fatty Acids (%)	SOP: TRANSGC.S based on AOCS Official Method Cd 14c-94	< 1	< 1	< 1

The unsaponifiable fraction of Ulkenia DHA oil is low, typically <1.4%, and consists primarily of sterols. Sterol components that are structurally similar to cholesterol, the primary sterol of animals, but are derived from non-animal sources, *i.e.*, plants, are collectively termed phytosterols (Kritchevsky, 1997). Such phytosterols have also been described in marine invertebrates (Piretti and Viviani, 1989). Phytosterols are common, naturally occurring constituents of the diet, with estimated daily intakes in the U.S. population ranging from 78 to 344 mg/day (Grundy and Mok, 1975; Nair *et al.*, 1984; Ling and Jones, 1995). Ling and Jones (1995) indicated an average phytosterol intake of 250 mg/day that could be doubled in

vegetarian consumers. However, estimating total phytosterol intake is dependent upon additional diet considerations, as Nair *et al.* (1984) reported daily phytosterol intakes of pure vegetarian Seventh Day Adventists was 89 mg/day and comparable to that of the non-vegetarian general population (78 mg/day). Phytosterol intakes were considerably higher in lacto-ovo vegetarians (344 mg/day) and non-vegetarian Seventh Day Adventists (231 mg/day). Nair *et al.* (1984) considered the dietary groups were better distinguished by the ratio of phytosterol intake to that of cholesterol, with higher ratios reported for both vegetarian groups compared with the non-vegetarian groups. In summarizing the reported intakes of phytosterols from various populations, Jones *et al.* (1997) indicated consumers of Western diets had intakes of 200 to 400 mg total phytosterols/day. Following comparison of 1957 and 1982 national diet survey data from Japan, Hirai *et al.* (1986) concluded daily per capita consumption of phytosterols has remained historically constant in Japanese diets, and reported intakes of 373 mg phytosterols/day for both survey years.

The predominant phytosterols consumed in the diet occur in various plant and seed oils, as β sitosterol, campesterol, stigmasterol, brassicasterol, and ergosterol, although greater than 40 plant sterols have been identified (Linscheer and Vergroesen, 1994; Kritchevsky, 1997). Phytosterols are poorly absorbed compared to cholesterol and the safety of dietary phytosterols has been well documented (Grundy and Mok, 1975; Linscheer and Vergroesen, 1994; Ling and Jones, 1995; Ayesh *et al.*, 1999; Baker *et al.*, 1999; Hepburn *et al.*, 1999; Waalkens-Berendsen *et al.*, 1999; Westrate *et al.*, 1999; Sanders *et al.*, 2000).

Representative lot analyses of Ulkenia DHA oil (Table 6) identified the main sterol components as cholesterol (approximately 20 to 30%), 24-ethyl-cholesta-5,7,22-trien-3-ol (7 dehydrostigmasterol) (approximately 28 to 34%), and 4-methyl-24-ethyl-cholesta-7,22-dien-3 ol (4-methyl-chondrillasterol) (approximately 14 to 19%). Exposure to the sterol components of Ulkenia DHA oil would not be considered novel as 4-methyl sterols are found in the normal metabolic pathway of cholesterol biosynthesis in man and they have been identified in several other food sources, including rice bran, fish and shellfish (Narumi and Takatsuto, 2000; Piretti and Vivani, 1989). 4-Methyl-24-ethyl-cholesta-7,22-dien-3-ol was previously described by Patterson, 1967 and Řezanka *et al.* (1986) in green algae (Chlorella). 24-Ethyl-stigmasta-5,7,22-trien-3-ol has been previously described as 7-dehydrostigmasterol by Smith and Korn (1968) in amoebae, and identified by Wright (1981) in algae, Dunaliella tertiolecta. Yates *et al.* (1992) indicated 24-ethyl-stigmasta-5,7,22-trien-3-ol had been reported in sponges, *Axinella cannabina,* and identified this sterol in celery cells as an intermediate in the synthesis of plant sterols. Furthermore, Pelletier *et al.* (1995) quantified levels of 492 mg 7-dehydrostigmasterol/ 100 g fat in phytosterols purified from the unsaponifiable fraction of soybean oil. Hence, the phytosterols in Ulkenia DHA oil would be considered to already occur in the diet, although quantitatively minor constituents of the consumed diet. Furthermore, as a very minor component of the Ulkenia DHA oil (<1.4%), safety of these sterol components have been confirmed through the toxicological evaluations undertaken on the Ulkenia DHA oil itself...

With typical sterol levels around or even below 1% in Ulkenia DHA oil, the level of exposure to sterols under the intended conditions of use of Ulkenia DHA oil would be minimal. Typical cholesterol levels are around 30 % of total sterols. Assuming a maximum intake of Ulkenia DHA oil of 7.9 g/day (based on 38% DHA in Ulkenia DHA oil contributing to 3 g/person/day), a total sterol content of 1% and 30% of sterols being cholesterol, the theoretical maximum daily cholesterol intake from Ulkenia DHA oil would be approximately 24 mg. Similarly, the theoretical maximum intake for phytosterols would be approximately 55 mg/day.

TAB 4

000090

ANALYSIS OF THE STABILITY OF ULKENIA DHA OIL (TAB 4)

Ulkenia DHA oil (containing 0.1% mixed tocopherols) was stored at 5, 25, and -35°C under nitrogen atmosphere and analyzed monthly and tri-monthly, respectively, for up to 36 months. The results of these analyses are provided in Tables A-8 through A-11. These studies indicate that Ulkenia DHA oil remains stable and within specification for at least 24 months when stored under inert atmosphere at temperatures ≤5°C and also when incorporated into dietary supplements.

Table A-8 Determination of the Stability of Ulkenia DHA oil Lot No. AA00103SE Following Storage at 5°C for 21 Months

Test	Specification	Time months									
		0	2	3	8	9	12	15	18	21	24
DHA % (w/w) in the oil	38 to 50	43.6	43.2	43.2	44.2	43.1	43.2	43.3	43.6	43.5	44.6
Peroxide value (meq./kg)	≤ 5	1.6	1.3	1.1	2.1	4.8	4.1	1.2	1.9	2.8	1.1

Table A-9 Determination of the Stability of Ulkenia DHA oil Lot No. AA00104S-4 Following Storage at 5°C for 36 Months

Test	Specification	Time months										
		0	9	12	15	18	21	24	27	30	33	36
DHA % (w/w) in the oil	38 to 50	42.1	41.6	40.7	41.5	41.3	42.9	42.0	40.9	40.7	40.8	38.8
Peroxide value (meq./kg)	≤ 5	0.7	0.8	1.7	1.3	1.3	1.6	1.5	4.3	3.3	2.5	3.7

Table A-10 Determination of the Stability of Ulkenia DHA oil Lot No. AA00104S-5 Following Storage at 25°C for 18 Months

Test	Specification	Time months						
		0	3	6	9	12	15	18
DHA % (w/w) in the oil	38 to 50	41.5	41.0	42.1	41.8	43.2	41.6	41.4
Peroxide value (meq./kg)	≤ 5	2.1	1.3	0.9	0.6	1.1	0.6	1.1

Table A-11	**Determination of the Stability of Ulkenia DHA oil Lot No. 110708 Following Storage at -35°C for 12 Months**					
Test	**Specification**	**Time months**				
		0	**3**	**6**	**9**	**12**
DHA (%) in the oil	38 to 50	42.0	40.4	39.4	38.9	39.1
Peroxide value (meq./kg)	≤ 5	2.4	0.8	1.0	0.8	2.8

Stability of Ulkenia DHA oil in dietary supplements

Product: 253 mg Lonza DHA CL in Vegetarian Capsules (110 mg DHA)

Capsule shell: Modified starch

Storage conditions: Room temperature (25 °C)

Relative humidity: 60%

Packed in blister

Stability testing of DHA rich dietary supplements was according to the International Conference on Harmonisation (ICH) stability testing guidelines (EMEA – The European Agency for the Evaluation of Medicinal Products).

Storage period	**DHA (% of total fatty acids)**	**DHA (% weight)**	**Peroxide Value (POV)**
Specification		38-50	≤ 5
0 month	43.5	39.6	3.1
3 months	43.7	39.8	3.5
6 months	43.2	41.7	5.0
12 months	43.7	39.6	2.6
18 months	43.0	39.2	3.9
24 months	43.3	39.0	3.9

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